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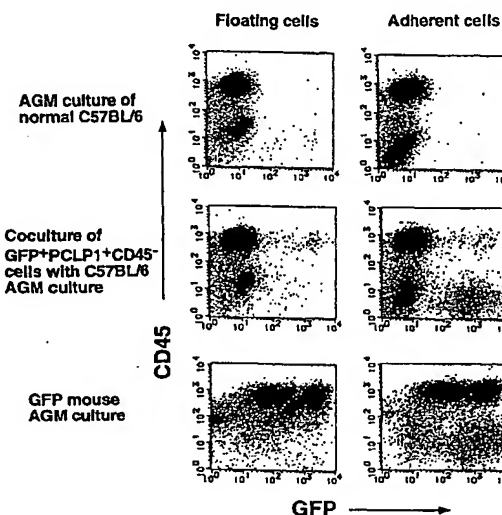
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## (54) METHOD FOR PREPARING CELL FRACTION CONTAINING HEMANGIOBLASTS

(57) Mouse PCLP1 was identified by expression cloning with the use of a monoclonal antibody against a surface antigen of a cell line derived from mouse AGM. By fractionating PCLP1-positive/CD45-negative cells and culturing them *in vitro*, it was clarified that these cells differentiate into endothelial-like cells, angioblast-like cells, and hematopoietic cells. By transferring the PCLP1-positive/CD45-negative cells into a mouse defective in the hematopoietic function, the hematopoietic system was reconstructed over a long period of time. These facts indicate that the PCLP1-positive/CD45-negative cells contain mammalian hemangioblasts capable of expressing the activity as long-term repopulating hematopoietic stem cells (LTR-HSC). The present invention provides a method for preparing a cell fraction containing hemangioblasts, the cell fraction prepared by the method, and use of this cell fraction.

Figure 7



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**Description****Technical Field**

5   **[0001]** The present invention relates to a marker molecule for hemangioblasts, method for preparing a cell fraction containing hemangioblasts using the marker molecule, cell fraction prepared by the method, and use of the cell fraction.

**Background Art**

10   **[0002]** Development of hematopoiesis proceeds through two distinct steps, i.e. primitive and definitive hematopoiesis. In mice, primitive hematopoiesis begins in the extraembryonic yolk sac at 7.5 days post coitum (dpc) in gestation, while definitive hematopoiesis, which is distinguished by enucleated erythrocytes, lymphopoiesis, and generation of long term repopulating hematopoietic stem cells (LTR-HSCs), originates from the intraembryonic aorta-gonad-mesonephros (AGM) region at 10.5 to 11.5 dpc (Muller, A. M. et al. (1994) *Immunity* 1, 291-301) (also reviewed by (Dzierzak, E. et al. (1998) *Immunol. Today* 19, 228-236; Keller, G. et al. (1999) *Exp. Hematol.* 27, 777-787). Within 1 to 3 days of their emergence, LTR-HSCs migrate from the AGM region to the fetal liver and then emigrate to the bone marrow just before birth. Lymphopoietic cells and multi-potential hematopoietic progenitors are also detected in the para-aortic splanchnopleura (P-Sp) region of mouse embryos at 7.5 to 9.5 dpc (Cumano, A. et al. (1996) *Cell* 86, 907-916; Delassus, S., and Cumano, A. (1996) *Immunity* 4, 97-106; Godin, I. et al. (1995) *Proc. Natl. Acad. Sci. USA* 92, 773-777), an intraembryonic site preceding the AGM region. However, LTR-HSCs, which are capable of repopulating lethally irradiated adult mice, have not been found in the P-Sp region. Interestingly, it was recently reported that LTR-HSCs can be detected in the yolk sac and the P-Sp region after transplantation into the livers of busulfan-treated newborn mice (Yoder, M. C. et al. (1997) *Immunity* 7, 335-344). Therefore, one speculation has been that LTR-HSCs generated in these sites lack homing capacity to the bone marrow and that the phenotypic differences in hematopoiesis between the yolk sac, the P-Sp region, and the AGM region can be mostly attributed to the supporting microenvironment. However, it still remains unknown how LTR-HSCs in the yolk sac acquire full repopulation activity.

25   **[0003]** Early in the last century, detailed observations of the early development of chick embryos led to the hypothesis that hematopoietic cells and endothelial cells arise from a common precursor termed the hemangioblast (Murray, P.D. F. (1932) *Proc. Roy. Soc. London* 11, 497-521; Sabin, F.R. (1920) *Contributions to Embryology* 9, 213-262) [also reviewed by (Wagner, R.C. (1980) *Adv. Microcirc.* 9, 45-75)]. In the last 5 years, a number of studies have provided evidence supporting this hypothesis. First, a series of elegant grafting experiments using chicks and quails demonstrated that the splanchnopleural mesoderm is able to generate hematopoietic cells and endothelium, while the paraxial mesoderm lacks this hematogenic capacity (Pardanaud, L. et al. (1996) *Development* 122, 1363-1371). Hematogenic activity in the former region is regulated by endoderm-derived cytokines such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor  $\beta$ 1 (TGF $\beta$ 1), whereas ectodermal factors such as epidermal growth factor (EGF) suppress it in the latter region (Pardanaud, L. and Dieterlen-Lievre, F. (1999) *Development* 126, 617-627). In the splanchnopleural mesoderm, cells expressing VEGF receptor 2 (VEGF-R2) were shown to form both hematopoietic and endothelial colonies (Eichmann, A. et al. (1997) *Proc. Natl. Acad. Sci. USA* 94, 5141-5146). Furthermore, endothelial cells in the dorsal aorta generated CD45<sup>+</sup> hematopoietic cells *in vivo* as evidenced by cell labeling experiments using Dil-labeled acetylated low density lipoprotein (Dil-Ac-LDL) (Jaffredo, T. et al. (1998) *Development* 125, 4575-4583).

40   **[0004]** While similar *in vivo* grafting experiments are not possible in mammalian systems, it was found that hematopoietic cells were clustered at the ventral wall of the dorsal aorta in a 5 week-old human embryo (Tavian, M. et al. (1996) *Blood* 87, 67-72). More recently, Tavian et al. showed that CD34<sup>+</sup> cells in the dorsal aorta and vitelline artery of human embryos are capable of generating hematopoietic cells *in vitro* (Tavian, M. et al. (1999) *Development* 126, 793-803). In mice, Nishikawa et al. recently showed that cells expressing Flk1 (mouse counterpart of VEGF-R2) and vascular endothelial cadherin (VECadherin) in the yolk sac and the P-Sp region of mouse embryos at 9.5 dpc gave rise to lymphohematopoietic cells *in vitro* (Nishikawa, S. et al. (1998) *Immunity* 8, 761-769). Similarly, Flk1<sup>+</sup> hematogenic endothelial cells were generated from ES cells *in vitro* (Choi, K. et al. (1998) *Development* 125, 725-732; Nishikawa, S. I. et al. (1998) *Development* 125, 1747-1757). The idea that putative hemangioblasts express Flk1 arose originally from the finding that knockout mice lacking Flk1 exhibited severe defects in both hematopoiesis and vasculogenesis in the yolk sac (Shalaby, F. et al. (1995) *Nature* 376, 62-66). Furthermore, Flk1-null cells did not contribute to definitive hematopoiesis (Shalaby, F. et al. (1997) *Cell* 89, 981-990), although a recent report suggested that a significant number of hematopoietic cells can be induced from Flk1-null ES cells *in vitro* (Schuh, A. C. et al. (1999) *Proc. Natl. Acad. Sci. USA* 96, 2159-2164).

55   **[0005]** In conformity with the critical role of TGF $\beta$ 1 in the induction of hematopoiesis and vasculogenesis in chick embryos, knockout mice deficient in TGF $\beta$ 1 also exhibited severe defects in both systems (Dickson, M. C. et al. (1995) *Development* 121, 1845-1854). Furthermore, gene disruption of the SCL/Tal-1 transcription factor caused defects in

both hematopoiesis and vasculogenesis which were similar to those of Flk1 knockout mice (Porcher, C. et al. (1996) Cell 86, 47-57; Visvader, J. E. et al. (1998) Genes Dev. 12, 473-479). Mutant zebrafish devoid of the SCL/Tal-1 gene also showed similar defects (Liao, E. C. et al. (1998) Genes Dev. 12, 621-626) and forced expression of SCL/Tal-1 in zebrafish embryos resulted in the overproduction of hematopoietic and vascular cells (Gering, M. et al. (1998) EMBO J. 17, 4029-4045). Taken together, these studies clearly demonstrate the presence of hemangioblasts, the common precursors of both hematopoietic and endothelial cells, in fish, avian, and mammalian embryos and that VEGF-R2/Flk1, TGF $\beta$ 1, and SCL/Tal-1 are essential for the development of hemangioblasts. However, the nature of hemangioblasts remains unexplored, in particular, there is yet no absolute evidence that LTR-HSCs are derived from hemangioblasts.

#### Disclosure of the Invention

**[0006]** An objective of the present invention is to provide a novel marker molecule for hemangioblasts, method for preparing a cell fraction containing hemangioblasts using the marker molecule, cell fraction prepared by the method, and use of the cell fraction.

**[0007]** The Inventors characterized the nature of mammalian hemangioblasts by using their AGM primary culture system, in which multipotential hematopoietic progenitor cells and endothelial-like cells expand *in vitro* (Mukouyama, Y. et al. (1998) Immunity 8, 105-114). In this culture, oncostatin M (OSM), a member of the IL-6 family of cytokines, is essential for the expansion of both cell types. Although OSM plays an essential role in the expansion of both cell populations, OSM does not directly stimulate the growth of hematopoietic progenitors in colony forming assays (data not shown). The inventors thus hypothesized that endothelial-like cells may contain hemangioblasts that produce hematopoietic progenitors in the AGM culture.

**[0008]** In order to isolate a novel marker molecule of endothelial-like cells in AGM culture, the present inventors prepared a monoclonal antibody against endothelial-like cells and conducted expression cloning using the antibody. As a result, the inventors succeeded in cloning a gene encoding the mouse counterpart (mouse PCLP1) corresponding to human and rabbit podocalyxin-like protein 1 (PCLP1).

**[0009]** Furthermore, the present inventors investigated properties of PCLP1<sup>+</sup>CD45<sup>-</sup> cells in the AGM region to find out the formation of both hematopoietic cells and endothelial cells from PCLP1<sup>+</sup>CD45<sup>-</sup> cells *in vitro*. By injecting PCLP1<sup>+</sup>CD45<sup>-</sup> cells into neonatal livers of busulfan-administered mice, the inventors further demonstrated that a plurality of hematopoietic cell lineages are produced over a long period of time *in vivo*, thereby proving for the first time that mammalian hemangioblasts are capable of constructing LTR-HSCs.

**[0010]** More specifically, PCLP1<sup>+</sup>CD45<sup>-</sup> cells thus selected exhibited endothelial-like morphology, incorporated acetylated low-density lipoprotein, and proliferated in response to OSM. In the co-presence of OP9 stromal cells together with VEGF and OSM, almost all the PCLP1<sup>+</sup>CD45<sup>-</sup> cells became positive for CD34, CD31 and VECadherin (Fig. 15), acquiring a capability to form a cellular network in the matrigel substrate (Fig. 16). These results indicate that PCLP1<sup>+</sup>CD45<sup>-</sup> cells have angioblast activity, and that OSM is essential for their proliferation and differentiation. On the other hand, Dil<sup>+</sup>CD45<sup>-</sup> endothelial-like cells (cf. examples) in the AGM primary culture generated hematopoietic cells *in vitro* (Fig. 1), and PCLP1<sup>+</sup>CD45<sup>-</sup> cells selected above similarly generated hematopoietic cells *in vitro* in the presence of hematopoietic growth factors and OP9 cells (Fig. 17). Most important finding was that PCLP1<sup>+</sup>CD45<sup>-</sup> cells reconstructed the entire hematopoietic system over a long period of time when transplanted into busulfan-treated neonatal mice (Figs. 18 to 21, and Table 1).

**[0011]** Thus, the present invention identifies PCLP1 as a novel cell marker for discriminating a cell fraction containing hemangioblasts and provides a method for preparing a cell fraction containing hemangioblasts using this marker.

**[0012]** As described above, although Nishikawa et al. previously reported the possibility that Flk1 and VECadherin could be marker molecules for hemangioblasts (Nishikawa, S. I. et al. (1998) Development 125, 1747-1757; Nishikawa, S. I. et al. (1998) Immunity 8, 761-769), expression level of PCLP1 is extremely high compared to those of Flk1 and VECadherin, which makes PCLP1 an excellent marker for first separating hemangioblasts.

**[0013]** More specifically, the present invention comprises:

- (1) a method for preparing a cell fraction containing hemangioblasts, wherein said method comprises separating cells comprising a PCLP1-positive phenotype,
- (2) the method according to (1), which further comprises separating cells comprising a CD45-negative phenotype,
- (3) the method according to (1) or (2), wherein said cells are separated from cells derived from the aorta-gonad-mesonephros (AGM) region,
- (4) a PCLP1-positive cell fraction containing hemangioblasts that is prepared by a method according to any one of (1) through (3),
- (5) the cell fraction according to (4), which generates or contains long-term repopulating hematopoietic stem cells (LTR-HSCs).
- (6) a cell composition containing the cell fraction according to (4) and a culture medium,

(7) a method for preparing a chimeric animal, wherein said method comprises transplanting the cell fraction according to (4),

(8) a chimeric animal transplanted with the cell fraction according to (4),

(9) the chimeric animal according to (8), wherein donor (transplanted cells)-derived blood cells can be reconstructed,

(10) the chimeric animal according to (8) or (9), wherein said animal is mouse,

(11) a DNA according to any one of the following (a) through (c), wherein the DNA encodes the mouse-derived PCLP1 protein:

(a) a DNA comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 1,

(b) a DNA encoding a protein comprising the amino acid sequence set forth in SEQ ID NO: 2, and

(c) a DNA encoding a protein comprising the amino acid sequence set forth in SEQ ID NO: 2 in which one or more amino acids are substituted, deleted, inserted, and/or added,

(12) a protein encoded by the DNA according to (11),

(13) a vector into which the DNA according to (11) has been inserted,

(14) a host cell carrying the vector according to (13),

(15) a method for preparing the protein according to (12), wherein said method comprises the steps of culturing the host cell according to (14) and collecting expressed proteins from said host cell or culture supernatant thereof,

(16) an antibody against PCLP1 protein, wherein the antibody is used for detecting or separating a cell fraction containing hemangioblasts,

(17) the antibody according to (16) that binds to a protein as defined in (12),

(18) a separation reagent for a cell fraction containing hemangioblasts, wherein said reagent comprises the antibody according to (16) or (17),

(19) an antibody that specifically binds to mouse-derived PCLP1 protein,

(20) an antibody that binds to a peptide comprising the amino acid residues at positions 1 to 405 in the amino acid sequence set forth in SEQ ID NO: 2,

(21) a peptide containing a partial sequence comprising at least 7 or more consecutive amino acid residues at positions 1 to 405 in the amino acid sequence set forth in SEQ ID NO: 2, and,

(22) a polynucleotide comprising at least 15 nucleotides, wherein the polynucleotide is complementary to DNA comprising the nucleotide sequence set forth in SEQ ID NO: 1 or to the complementary strand thereof, and is used in the amplification and detection of the expression of the DNA according to (11), or in the expression control of the DNA.

**[0014]** Besides mouse PCLP1, "PCLP1s" of the present invention includes, unless the origin is otherwise specified, PCLP1s derived from vertebrates including human or rabbit podocalyxin-like protein 1 (PCLP1) (Kershaw, D. B. et al. (1997) J. Biol. Chem. 272, 15708-15714; Kershaw, D. B. et al. (1995) J. Biol. Chem. 270, 29439-29446), preferably PCLP1 derived from mammals. Similarly, "CD45s" of the present invention includes CD45s derived from vertebrates, preferably CD45s derived from mammals.

**[0015]** Herein, "hemangioblasts" refers to cells capable of generating both endothelial cells and hematopoietic cells.

**[0016]** "Endothelial cells" refers to adherent cells showing endothelial cell morphology, namely polygonal morphology when cultured *in vitro*, which have the activity to incorporate acetylated low density lipoprotein. In this invention, more preferably, endothelial cells can proliferate in response to a stimulation by OSM. Even more preferably, in endothelial cell differentiation culture systems, cells positive for hematopoietic cell markers such as CD34, CD31, and VECadherin could be generated when co-cultured with OP9 stromal cells in the presence of VEGF, OSM, etc. Also, still more preferably, endothelial cells can give rise to a cellular network formation in matrigel plate assays. These properties can be assayed according to methods described in Examples.

**[0017]** "Hematopoietic cells" refers to cells expressing a hematopoietic cell phenotype, meaning spherical non-adherent cells with a CD45-or Ter119-positive phenotype. During their differentiation, hematopoietic cells specifically express combinations of B220, Mac-1, Gr-1, Thy-1, CD4, CD8, etc. as lineage markers.

**[0018]** "Hematopoietic cells" includes "hematopoietic stem cells". These hematopoietic stem cells are preferably CD45-positive. Preferably, when co-cultured with OP9 cells in the presence of hematopoietic growth factors including SCF, interleukin (IL)-3, erythropoietin (EPO), etc., hematopoietic stem cells generate myeloid (e.g. Mac-1/Gr-1-positive), lymphoid (e.g. B220/Thy-1-positive) or erythroid (e.g. Ter119-positive) cells. Alternatively, hematopoietic stem cell phenotype can be confirmed by reconstruction of hematopoietic stem cells or blood cells derived from transplanted cells that are transplanted into animals lacking hematopoietic functions.

**[0019]** In this invention, "long term repopulating hematopoietic stem cells (LTR-HSCs)" refers to hematopoietic stem cells capable of reconstructing hematopoiesis over a long duration.

**[0020]** The present invention provides a method for preparing a cell fraction containing hemangioblasts, characterized by separating cells expressing a PCLP1 (podocalyxin-like protein 1)-positive (described as PCLP<sup>+</sup>) phenotype.

**[0021]** As cells used in the aforementioned separation, tissues and cells presumed to contain hemangioblasts, or hemangioblast cultures, may be used. Most preferable cells are those derived from the aorta-gonad-mesonephros (AGM) region. These cells may be vertebrate-derived, preferably mammalian cells (for example, cells from rodents), or may be human cells. In mice, cells derived from the intraembryonic AGM region at 10 to 11.5 dpc, which corresponds to the AGM region of a 4 to 5 week-old human embryo, are preferable in particular.

**[0022]** Moreover, since it has been suggested that dorsal aorta-associated endothelial cells contain the budding site of hematopoietic cells (Tavian, M. et al. (1996) *Blood* 87, 67-72; Tavian, M. et al. (1999) *Development* 126, 793-803), hemangioblasts are highly likely to exist in this region. Furthermore, the present inventors indicate the possibility of the presence of hemangioblasts in the genital ridge region (F, I, O in Fig. 10 to 11). Therefore, cells derived from these regions can be also used.

**[0023]** OSM-dependant proliferation of hematopoietic precursor cells and endothelial-like cells were also observed in a primary culture of the P-Sp region of an embryo at 9.5 dpc similarly to a culture of AGM derived from a mouse embryo at 11.5 dpc. Although such a proliferation was not observed in the yolk sac at 9.5 dpc, PCLP1<sup>+</sup>CD45<sup>-</sup> cells were present in both yolk sac and P-Sp region (data not shown). Therefore, cells derived from the yolk sac and P-Sp region are highly likely to contain hemangioblasts. Therefore, cells such as these can also be used in the separation in this invention.

**[0024]** It is also possible to isolate PCLP1<sup>+</sup> cells or PCLP1<sup>+</sup>CD45<sup>-</sup> cells using ES-derived cells. Generation and proliferation of LTR-HSC from ES cells are also important in the application of human ES cells. Furthermore, recently, CD34<sup>+</sup> blood progenitor endothelial cells were found in peripheral blood, which were shown to be bone marrow-derived (Asahara, T. et al. (1997) *Science* 275, 964-967; Takahashi, T. et al. (1999) *Nat. Med.* 5, 434-438). Very recently, Bjornson et al. reported that LTR-HSCs are derived from cultured neural stem cells *in vivo* (Bjornson, C. R. et al. (1999) *Science* 283, 534-537). Therefore, it is likely that hemangioblasts exist in these tissues besides embryonic hematopoietic sites. In this invention, such cells may also be also used for isolating PCLP1<sup>+</sup> cells or PCLP1<sup>+</sup>CD45<sup>-</sup> cells.

**[0025]** Neonatal and adult tissues, for example, umbilical cord and bone marrow can be also used. Separation of hemangioblasts using the method of this invention is extremely significant in the clinical application of these cells.

**[0026]** Separation of PCLP1<sup>+</sup> cells can be performed, for example, by cell sorting as described in Examples using anti-PCLP1 antibody.

**[0027]** In this invention, in order to obtain cell fractions containing hemangioblasts in a high concentration, it is preferable to separate cells further expressing the CD45<sup>-</sup> phenotype in addition to the PCLP1<sup>+</sup> phenotype. Separation of cells having the PCLP1<sup>+</sup>CD45<sup>-</sup> phenotype can be performed, for example, according to methods described in Examples. PCLP1<sup>+</sup> cell fraction or PCLP1<sup>+</sup>CD45<sup>-</sup> cell fraction containing hemangioblasts can be further subdivided using different cell markers.

**[0028]** For example, in the present invention, it may be useful to fractionate CD34<sup>+</sup> cells from PCLP1<sup>+</sup> cell fraction or PCLP1<sup>+</sup>CD45<sup>-</sup> cell fraction. In Examples, the inventors have identified PCLP1 as a marker for hemangioblasts. PCLP1 is a highly glycosylated protein with some similarity to CD34, a conventional marker for LTR-HSCs. Interestingly, both PCLP1 and CD34 are ligands for L-selectin in the lymphocyte-high endothelium venule and contain conserved amino acid sequences in their cytoplasmic regions (Sasseti, C. et al. (1998) *J. Exp. Med.* 187, 1965-1975), suggesting an overlapping function between the two molecules. In fact, both PCLP1 and CD34 are expressed in the dorsal aortic endothelium and the genital ridge region (Figure 10 and 11) and as much as 91% of CD34<sup>+</sup> cells in the AGM region also express PCLP1 (Figure 8, right). In chicken, thrombomucin, an avian counterpart of PCLP1, is expressed in hematopoietic progenitors and thrombocytes (McNagny, K. M. et al. (1997) *J. Cell Biol.* 138, 1395-1407), however no avian CD34 has been identified. Therefore, the functions, if any, of CD34 might be compensated by PCLP1. Sanchez et al. showed that LTR-HSCs in the AGM region are CD34<sup>+</sup>c-Kit<sup>+</sup> (Sanchez, M. J. et al. (1996) *Immunity* 5, 513-525) and LTR-HSCs in the yolk sac and P-Sp region were also found in the CD34<sup>+</sup>c-Kit<sup>+</sup> fraction (Yoder, M. C. et al. (1997) *Immunity* 7, 335-344). Since PCLP1<sup>+</sup>CD34<sup>+</sup>CD45<sup>-</sup> cells (12% of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells) exist in the AGM region (Figure 8, right), the LTR-HSC activity found in the CD34<sup>+</sup> fraction might represent, in part, that of the hemangioblasts. Thus, the PCLP1<sup>+</sup>CD34<sup>+</sup>CD45<sup>-</sup> cells in the AGM region are important as a cellular fraction containing hemangioblasts, and are likely to contain a high concentration of LTR-HSCs that are capable of reconstructing, in particular, hematopoietic systems.

**[0029]** Furthermore, in this invention, it may be also useful to fractionate Flk1-positive cells from the PCLP1<sup>+</sup> cell fraction or the PCLP1<sup>+</sup>CD45<sup>-</sup> cell fraction. Recently, Nishikawa et al. demonstrated the hematopoietic activity in an Flk1<sup>+</sup>VECadherin<sup>+</sup>CD45<sup>-</sup> cell population derived from the yolk sac and P-Sp region of mouse embryo at 9.5 dpc (Nishikawa, S. I. et al. (1990) *Immunity* 8, 761-769). Studies on ES differentiation *in vitro* and Flk1-knockout mice also indicated that putative hemangioblasts express Flk1 (Choi, K. et al. (1998) *Development* 125, 725-732; Nishikawa, S. I. et al. (1998) *Development* 125, 1747-1757; Shalaby, F. et al. (1995) *Nature* 376, 62-66). In accordance with these results, one fraction (12%) of PCLP1<sup>+</sup>CD45<sup>-</sup> cells in the AGM region expressed Flk1 (Fig. 9, left), and PCLP1<sup>+</sup>CD45<sup>-</sup>

cells cultured in the presence of OSM were all Flk1-positive (Fig. 14, below). In contrast, frequency of VECadherin<sup>+</sup> cells in the PLCP1<sup>+</sup>CD45<sup>-</sup> cell population in the AGM region was very low (3%) (Fig. 9, right). From these findings, PLCP1<sup>+</sup>CD45<sup>-</sup>Flk1<sup>+</sup> cells in AGM region are important as a cell fraction containing hemangioblasts.

**[0030]** Cell fractions prepared in the present invention can be cultured or stored in an appropriate medium, which may be supplemented with serum, growth/differentiation factors, etc. As a medium, for example, DMEM containing 15% FCS supplemented with OSM and SCF, or the like can be used.

**[0031]** Among cells contained in cell fractions of this invention, long-term repopulating hematopoietic stem cells (LTR-HSCs) are important in particular. Presence of LTR-HSC in a cell fraction can be confirmed by transplanting the cell fraction into an animal made deficient in hematopoietic functions to prepare a chimeric animal, and assaying the capability of the cells to reconstruct the hematopoietic system.

**[0032]** As described in Examples, chimeric animals can be prepared by transplanting the cell fraction of this invention into livers of neonatal mice in which the hematopoietic function has been destructed by busulfan administration.

**[0033]** There is no restriction on the type of animals used for preparing chimeras, examples being mice, rabbits, other large-sized animals, etc.

**[0034]** Establishment of chimerism can be confirmed by examining the post-transplantational generation of donor-derived blood cells.

**[0035]** In chimeric animals transplanted with the cell fraction of this invention containing LTR-HSCs, donor (transplanted cells)-derived LTR-HSC is generated in the recipient reconstructing the hematopoietic system. Inclusion of LTR-HSC in transplanted cells can be confirmed by the appearance of donor-derived lymphoid, myeloid, and erythroid cells above the detection limit (for example, 1% or more) in recipients. In addition, occurrence of a long-term reconstruction of hematopoiesis can be confirmed by detecting donor-derived blood cells at least 60 days, more preferably 180 days after the transplantation of LTR-HSC.

**[0036]** PLCP1<sup>+</sup>CD45<sup>-</sup> cells and recipients transferred with the cells are useful in the screening of drugs that control the proliferation and differentiation of hemangioblasts. For example, by adding a test compound to PLCP1<sup>+</sup>CD45<sup>-</sup> cells in culture, effects of the compound on the proliferation, differentiation, hematopoietic function, and the like of the cells can be examined. By administering a test compound to a recipient (for example, mouse) transplanted with PLCP1<sup>+</sup>CD45<sup>-</sup> cells, effects of the compound on hematopoiesis of the recipient can be also investigated.

**[0037]** Furthermore, the present invention relates to an antibody against the PCLP1 protein used for the detection or separation of cell fractions containing hemangioblasts. This invention also relates to the use of an antibody raised against the PCLP1 protein in the detection or separation of cell fractions containing hemangioblasts. Such an antibody binds to the cell surface PCLP1 protein. Such an antibody is suitably prepared by using the extracellular domain of the PCLP1 protein as an antigen, or cells expressing the PCLP1 protein as an immunogen as described in Examples.

**[0038]** There is no particular restriction on the animal species from which the PCLP1 protein that is used as the immunogen is derived, and it may be human (J. Biol. Chem. 272: 15708-15714 (1997)), mouse, rat (Accession number: ABO20726), rabbit (J. Biol. Chem. 270: 29439-29446 (1995)), chicken (J. Cell Biol. 138: 1395-1407 (1997)), or another vertebrate. Antibodies can be prepared according to methods well-known in the field. For example, in the case of a monoclonal antibody, it may be prepared by the rat foot-pad immunization method (Hockfield, S. et al. (1993) "Selected Methods for Antibody and Nucleic Acid Probes", Volume 1 (New York: Cold Spring Harbor Laboratory Press)), etc.

**[0039]** Such an antibody can be appropriately combined with buffers and stabilizers to make a reagent for detecting or separating cell fractions containing hemangioblasts. This antibody may also become a test reagent for cell fractions containing hemangioblasts. The antibody may be fluorescence-labeled.

**[0040]** The present invention also provides the mouse PCLP1 protein. The mouse PCLP1 gene was isolated by expression cloning using a monoclonal antibody against the cell surface antigen on a mouse AGM region-derived cell line (LO cells). The nucleotide sequence of cDNA encoding the mouse PCLP1 protein isolated by the present inventors is set forth in SEQ ID NO: 1, and the amino acid sequence of the mouse PCLP1 protein encoded by the cDNA is set forth in SEQ ID NO: 2, respectively. PLCP1<sup>+</sup> cell fractions contain hemangioblasts capable of generating endothelial cells and hematopoietic cells.

**[0041]** Proteins of this invention include, as long as they can serve as marker molecules for hemangioblasts (for example, proteins with overlapping antigenicities), not only the wild type PCLP1 protein (SEQ ID NO: 2), but also proteins structurally analogous thereto. Such structurally analogous proteins include mutants of the wild type mouse PCLP1 protein. Whether antigenicity is overlapped or not can be determined by immunizing a recipient animal with the wild type mouse PCLP1 protein as antigen and examining whether the antibody thus produced has a reactivity toward a protein of interest. Alternatively, the above test can be performed by preparing an antibody against a protein of interest to determine the reactivity of the protein with the wild type mouse PCLP1 protein.

**[0042]** Such proteins include not only naturally occurring mutants but also artificially prepared mutants that can be produced by those skilled in the art, for example, using the known method for mutagenesis Methods known to those skilled in the art for modifying amino acids in proteins are exemplified by Kunkel's method and PCR.

**[0043]** In artificial alteration of amino acids in proteins, the number of amino acid residues to be altered is usually 30

or less, preferably 10 or less, and more preferably 5 or less. An amino acid having properties similar to those of the amino acid to be substituted is preferably used for substitution. For example, since Ala, Val, Leu, Ile, Pro, Met, Phe, and Trp are all classified into the non-polar amino acid group, they are considered to have similar properties. Non-charged amino acids include Gly, Ser, Thr, Cys, Tyr, Asn, and Gln. Acidic amino acids include Asp and Glu, while basic amino acids include Lys, Arg, and His.

**[0044]** The protein of this invention can be prepared as either a natural protein or a recombinant protein utilizing gene recombination techniques. Natural proteins can be prepared by, for example, subjecting extracts from tissues and cells that are presumed to express mouse PCLP1 protein (for example, LO cells, embryo cells in AGM region, etc.) to affinity chromatography using the above-described antibody to the mouse PCLP1 protein. On the other hand, recombinant proteins may be prepared by culturing cells transformed with DNA encoding the mouse PCLP1 protein, allowing the transformants to express the protein, and recovering the protein as described below. Proteins of this invention can be fused to peptide tags and other proteins. Such fusion proteins can be useful for facilitating the purification and detection of proteins.

**[0045]** The present invention also includes partial peptides of the protein of this invention. Partial peptides of this invention include peptides containing partial sequences comprising at least 7 or more consecutive amino acid residues, preferably 8 amino acid residues or more, and more preferably 9 amino acid residues or more in the region specific (at positions 1 to 405 of the amino acid sequence set forth in SEQ ID NO: 2) to mouse PCLP1. An antibody specifically binding to the mouse-derived PCLP1 protein can be obtained by preparing the antibody using the above-described partial peptides as antigens. Examples of partial peptides of this invention are, for example, those of the N-terminal region of proteins of this invention (for example, SEQ ID NO: 2) and intracellular domain thereof, and these can be used in the antibody preparation. Furthermore, in the mouse PCLP1 protein (SEQ ID NO: 2), peptides containing the regions comprising 12 amino acid residues at positions 440 to 451, 10 amino acid residues at positions 464 to 473, or 4 amino acid residues at positions 500 to 503 in the intracellular domain analogous to CD34 are considered to be useful as partial peptides to search functions common to both CD34 and PCLP1. Partial peptides of this invention can be produced by, for example, genetic engineering techniques, known peptide synthesis methods, or by digestion of the protein of this invention with appropriate peptidases.

**[0046]** This invention also relates to DNAs encoding the proteins of the invention. DNAs encoding the protein of this invention are not particularly limited as long as they can encode the proteins of this invention, including cDNAs, genomic DNAs, and synthetic DNAs. DNAs having any desired nucleotide sequence based on the degeneracy of genetic code are also included in this invention as long as they can encode the proteins of this invention.

**[0047]** cDNAs encoding the proteins of this invention can be screened, for example, by labeling cDNA of SEQ ID NO: 1 or segments thereof, RNAs complementary to them, or synthetic oligonucleotides comprising partial sequences of the cDNA with <sup>32</sup>P, etc., and hybridizing them with a cDNA library derived from tissues (e.g., cells derived from AGM region of embryo, etc.) expressing the proteins of this invention. Alternatively, such cDNAs can be cloned by synthesizing oligonucleotides corresponding to nucleotide sequences of these cDNAs, and amplifying them by PCR with cDNA derived from suitable tissues (e.g. cells derived from AGM region of embryo, etc.) as a template. genomic DNA can be screened, for example, by labeling cDNA of SEQ ID NO: 1 or segments thereof, RNAs complementary to them, or synthetic oligonucleotides comprising partial sequences of the cDNA with <sup>32</sup>P, etc., and hybridizing them with a genomic DNA library. Alternatively, the genomic DNA can be cloned by synthesizing oligonucleotides corresponding to nucleotide sequences of these cDNAs, and amplifying them by PCR with genomic DNA as a template. Synthetic DNAs can be prepared, for example, by chemically synthesizing oligonucleotides comprising partial sequences of cDNA of SEQ ID NO: 1, annealing them to form a double strand, and ligating them with DNA ligase.

**[0048]** These DNAs are useful for the production of recombinant proteins. A protein of this invention can be prepared as a recombinant protein by inserting the DNA encoding the protein (e.g. DNA of SEQ ID NO: 1) into an appropriate expression vector, transforming suitable cells with the vector, culturing the transformants, and purifying the expressed protein from the transformants or their culture supernatant.

**[0049]** There is no limitation on the host and vector to be used, and it is possible to use a prokaryotic or eukaryotic system. Specifically, for example, COS7 cells and the pME18S vector can be used. A vector can be introduced into cells by a known method, for example, DEAE-dextran method (Blood 90: 165-173, 1997) for mammalian cells.

**[0050]** Recombinant proteins expressed in host cells can be purified by known methods. The protein of this invention expressed in the form of a fusion protein, for example, with a histidine residue tag or glutathione-S-transferase (GST) attached to the N-terminus can be purified by a nickel column or a glutathione sepharose column, etc.

**[0051]** The present invention also provides a polynucleotide containing at least 15 nucleotides complementary to the DNA comprising the nucleotide sequence of SEQ ID NO: 1 or to the complementary strand thereof, which is used to amplify a DNA encoding a mouse PCLP1 protein, detect the expression, or regulate the expression. Herein, the term "complementary strand" refers to one strand of a double strand polynucleotide comprising A:T (A:U) and G:C base pairs, when viewed against the other strand. Furthermore, "complementary" means not only when a nucleotide sequence is completely complementary to a continuous nucleotide sequence with at least 15 nucleotides, but also



when there is an identity of at least 70%, preferably at least 80%, more preferably 90%, and much more preferably 95% or more at the nucleotide sequence level. The identity can be determined by BLAST. Polynucleotides include DNA and RNA. Nucleotide derivatives can also be included.

[0052] Such polynucleotides include probes, primers, nucleotides or nucleotide derivatives (e.g. antisense oligonucleotides and ribozymes, etc.) , which can specifically hybridize with DNAs encoding mouse PCLP1 proteins, or DNAs complementary to said DNAs.

[0053] cDNAs encoding the proteins of this invention or oligonucleotides comprising partial sequences thereof can be used for cloning genes and cDNAs encoding the proteins of this invention, or amplifying them by PCR. They can also be used as, for example, probes for Northern blot analyses, or as primers for RT-PCR to detect or quantify the expression. The cDNAs and oligonucleotides can also be utilized for detecting polymorphism or an aberration (gene diagnosis, etc.) of the gene or cDNA by the restriction fragment length polymorphism (RFLP) method, single strand DNA conformation polymorphism (SSCP) method, etc. It is also possible to suppress the expression of the mouse PCLP1 protein using an antisense polynucleotide.

[0054] The present invention also provides an antibody specific to mouse PCLP1. The antibody may be prepared by extracting sequences specific to the mouse PCLP1 protein based on a comparison of the amino acid sequence of mouse PCLP1 protein with those of PCLP1 proteins derived from other vertebrates, and immunizing appropriate animals with peptides having said sequences as described above. There is no limitation in the region of these peptides that is used for the immunization as long as they are immunogenic. Antibodies of this invention include, for example, those binding to peptides comprising the amino acid residues at positions 1 to 405 set forth in SEQ ID NO: 2.

[0055] Antibodies thus prepared can be utilized, besides in cell sorting, for example, in the affinity purification of proteins of this invention, test and diagnosis of disorders in patients or in disease models having disorders caused by an expressional aberration, or structural abnormality of a protein of this invention, and the like, and also in the detection of the expression levels of the inventive proteins. More specifically, aberrations in the expression and structure of proteins of this invention can be tested and diagnosed by using methods such as FACS, Western blotting, immunoprecipitation, ELISA, immunohistochemical technique, etc. to detect the proteins in samples extracted from tissues, blood, cells, or tissue segments.

[0056] Prior arts and other references cited in the present specification are all incorporated herein as a part thereof.

#### Brief Description of the Drawings

[0057] Figure 1 is a diagram representing generation of hematopoietic cells from endothelial-like cells in an AGM culture.

[0058] Endothelial-like cells generated in a day 6 AGM primary culture were pulse labeled with Dil-Ac-LDL for 6 h. Dil\*CD45<sup>-</sup> cells (RI fraction) were then isolated by FACS and inoculated into an unlabeled day 6 AGM culture. After a 4-day chase culture, both floating and adherent cells were analyzed by FACS. Dil\*CD45<sup>+</sup> hematopoietic cells (shown by "}") were generated from the Dil-labeled endothelial-like cells. CD45<sup>+</sup> cells in the adherent cell fraction are likely to be cobble stone-forming cells and cells attached to the stromal cell layer.

[0059] Figure 2 is a diagram representing cloning and expression of mouse PCLP1.

(A) FACS staining of LO cells with 10B9 monoclonal antibody (shaded peak) or isotype control (blank peak).

(B) FACS staining of COS7 cells transfected with mouse PCLP1 cDNA (shaded peak) or mock vector (blank peak) with 10B9 antibody.

[0060] Figure 3 is a diagram representing an alignment of amino acid sequences of mouse, human, and rabbit PCLP1, and avian thrombomucin. The underline and double-underlines represent signal peptides and transmembrane domains, respectively. In human PCLP1, the reported signal peptide contains two extra amino acid residues in addition to the underlined residues (...LP).

[0061] Figure 4 is a photograph representing the result of Northern blot analysis of PCLP1 mRNA in various mouse adult tissues and LO cells. PolyA<sup>+</sup> RNA (1 µg) was loaded in each lane.

[0062] Figure 5 is a photograph representing the expression of PCLP1 in an AGM culture.

(A) Morphological appearance of endothelial-like cells in the AGM primary culture on day 6.

(B, C) Immunostaining of endothelial-like cells in the AGM culture with isotype control (B) or 10B9 anti-PCLP1 antibody (C). Original magnification: (A) 100 x; (B, C) 200 x.

[0063] Figure 6 is a diagram representing the expression of PCLP1 and CD45 in an AGM culture.

Represents a FACS analysis of the total cells in the AGM culture on day 6 with anti-PCLP1 and anti-CD45 antibodies. R2 gated cells were sorted as a PCLP1\*CD45<sup>-</sup> fraction for the co-culture experiment shown in Figure 7.



**[0064]** Figure 7 is a diagram representing the result of the FACS analysis of the floating cells and adherent cells in a co-culture (middle panel) where the PCLP1<sup>+</sup>CD45<sup>-</sup> cells from the AGM culture of GFP mice were sorted on day 6 and co-cultured with the AGM culture of normal mice for 4 more days. Upper and bottom panels are negative (AGM culture of normal mice) and positive (AGM culture of GFP mice) controls for the detection of GFP, respectively. Note that GFP<sup>+</sup>CD45<sup>+</sup> cells are generated from PCLP1<sup>+</sup>CD45<sup>-</sup> cells in the co-culture.

**[0065]** Figure 8 is a diagram representing the result of FACS analyses of the AGM region of mouse embryos.

(A) Expression of PCLP1 and CD45 in cells from the AGM region. A single cell suspension prepared from the AGM regions of mouse embryos at 11.5 dpc was stained with anti-CD45 antibody and anti-PCLP1 antibody or isotype control and analyzed by FACS.

(B) Expression of CD31 and CD34 in the CD45<sup>-</sup> cell population. The AGM-derived cells were stained with anti-PCLP1 antibody and anti-CD45 antibody together with either anti-CD34 or anti-CD31 antibodies. FACS profiles of PCLP1 and CD34 or CD31 in the CD45<sup>-</sup> cell fraction are shown. Note that most of CD34<sup>+</sup> and CD31<sup>+</sup> cells are included in the PCLP1<sup>+</sup>CD45<sup>-</sup> cell fraction.

Figure 9 is a diagram representing the result of FACS analysis of the AGM region of mouse embryos.

Shows the expression of Flk1 and VECadherin in the CD45<sup>-</sup> cell population. The AGM-derived cells were stained with anti-PCLP1 and anti-CD45 antibodies together with either anti-Flk1 antibody (C) or anti-VECadherin antibody (D). Expression patterns of PCLP1 and Flk1

(C) or VECadherin (D) in the CD45<sup>-</sup> cell fraction are presented.

**[0066]** Figure 10 is a photograph representing an expression of PCLP1 in the AGM region of a mouse embryo.

(A to I) Paraffin sections of the AGM region of a mouse embryo at 11.5 dpc were stained immunohistochemically with isotype control (A to C), anti-PCLP1 (D to F), or anti-CD34 (G to I) antibodies. Expression of PCLP1 and CD34, shown in brown, mostly overlap in the aorta (E, H) and genital ridge regions (F, I).

**[0067]** Figure 11 is a photograph representing an expression of PCLP1 in the AGM region of a mouse embryo.

(J to O) Paraffin sections of the AGM region were subjected to in situ hybridization using sense (J to L) or anti-sense (M to O) cRNA to the mouse PCLP1 cDNA as a probe. Specific signals are shown in dark blue. Original magnification was 40 x (left panels). Aorta and genital ridge regions are further enlarged in middle and right panels, respectively.

**[0068]** Figure 12 is a diagram representing differentiation of PCLP1<sup>+</sup>CD45<sup>-</sup> cells into endothelial cells.

**[0069]** Shows the FACS analysis of the cells in the AGM regions of mouse embryos at 11.5 dpc with anti-PCLP1 and anti-CD45 antibodies. In lower panels, sorted PCLP1<sup>+</sup>CD45<sup>-</sup> cells (R1 gate) or PCLP1<sup>-</sup>CD45<sup>-</sup> cells (R2 gate) were reanalyzed, respectively.

**[0070]** Figure 13 is a photograph representing differentiation of PCLP1<sup>+</sup>CD45<sup>-</sup> cells into endothelial cells.

**[0071]** Morphological appearance of the sorted PCLP1<sup>+</sup>CD45<sup>-</sup> cells from the AGM region after 6 days in culture with SCF and OSM.

**[0072]** Figure 14 is a diagram representing differentiation of PCLP1<sup>+</sup>CD45<sup>-</sup> cells into endothelial cells.

**[0073]** Top panel: Incorporation of Dil-labeled acetylated LDL into PCLP1<sup>+</sup>CD45<sup>-</sup> cells after 6 days in culture. Shaded and blank peaks represent FACS patterns of cells incubated with or without Dil-acetylated LDL, respectively.

**[0074]** Bottom panel: Expression of Flk1 on the PCLP1<sup>+</sup>CD45<sup>-</sup> cells after 6 days in culture. Cells were stained with anti-Flk1 (shaded peak) or isotype control (blank peak) antibody and subjected to FACS analysis.

**[0075]** Figure 15 is a diagram representing differentiation of PCLP1<sup>+</sup>CD45<sup>-</sup> cells into endothelial cells.

**[0076]** Induction of the expression of various endothelial cell markers in PCLP1<sup>+</sup>CD45<sup>-</sup> cells after co-culture with OP9 stromal cells for 10 days in the presence of OSM, VEGF, and bFGF. OP9 cells were gated out by the forward scatter window and the remaining major cell fraction was stained with antibodies as indicated. Left panels demonstrate FACS patterns of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells after 6 days in culture in the absence of OP9. Blank and light shaded peaks show staining patterns of isotype control and specific antibodies, respectively.

**[0077]** Figure 16 is a photograph representing differentiation of PCLP1<sup>+</sup>CD45<sup>-</sup> cells into endothelial cells.

Shows vascular network formation of the endothelial cells. After 10 days in co-culture of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells with OP9, cells were placed on the matrigel and cultured for 12 hours.

**[0078]** Figure 17 is a diagram representing generation of hematopoietic cells from PCLP1<sup>+</sup>CD45<sup>-</sup> cells *in vitro*.

**[0079]** The PCLP1<sup>+</sup>CD45<sup>-</sup> cells isolated from the AGM region were co-cultured with OP9 stromal cells in the presence of SCF, bFGF, OSM, LIF, IL-3, and EPO for 10 days. Floating cells were double-stained with two anti-hematopoietic lineage marker antibodies or isotype controls as indicated and subjected to FACS analysis.

**[0080]** Figure 18 is a diagram representing induction of long-term repopulating hematopoietic stem cells *in vivo* from PCLP1<sup>+</sup>CD45<sup>-</sup> hemangioblasts.

**[0081]** GFP<sup>+</sup>PCLP1<sup>+</sup>CD45<sup>-</sup> cells were isolated from the AGM region of the GFP transgenic mice and  $1.7 \times 10^5$  cells were injected into the liver of neonatal C57BL/6 mice at 36 h after birth. After 6 months, peripheral blood was taken from one mouse (10B3 mouse) and mononuclear cells were stained with various antibodies against hematopoietic

lineage markers as indicated. FACS patterns of C57BL/6 and GFP mouse are shown as negative and positive controls, respectively. Note the higher contribution of the donor-derived cells in the hematopoietic system of the 10B3 mouse.

[0082] Figure 19 is a diagram representing the induction of long-term repopulating hematopoietic stem cells from PLCP1<sup>+</sup>CD45<sup>+</sup> hemangioblasts *in vivo*. Spleen was extracted from a similarly transplanted mouse as in Fig. 18, and mononuclear cells were stained with various antibodies against hematopoietic lineage markers as indicated.

[0083] Figure 20 is a diagram representing the induction of long-term repopulating hematopoietic stem cells from PLCP1<sup>+</sup>CD45<sup>+</sup> hemangioblasts *in vivo*. Bone marrow was collected from a similarly transplanted mouse as in Fig. 18, and mononuclear cells were stained with various antibodies against hematopoietic lineage markers as indicated.

[0084] Figure 21 is a diagram representing the induction of long-term repopulating hematopoietic stem cells from PLCP1<sup>+</sup>CD45<sup>+</sup> hemangioblasts *in vivo*. Thymus was extracted from a similarly transplanted mouse as in Fig. 18, and mononuclear cells were stained with various antibodies against hematopoietic lineage markers as indicated. Analysis of thymus from 10B3 mouse was first performed gating GFP<sup>+</sup> thymocytes to analyze the expression of CD4 and CD8.

#### Best Mode for Carrying out the Invention

[0085] The present invention will be explained in detail below with reference to Examples, but it is not to be construed as being limited thereto.

[0086] Timed pregnant C57BL/6 mice were purchased from Nihon SLC (Hamamatsu, Japan). GFP transgenic mice (Okabe, M. et al. (1997) FEBS Lett. 407, 313-319) were maintained and mated in an animal facility. The time at midday (12:00) was taken to be 0.5 dpc for the plugged mice. As previously described (Mukouyama, Y. et al. (1998) Immunity 8, 105-114), AGM regions were dissected from mouse embryos at 11.5 dpc and a single cell suspension was subjected to primary culture.

[0087] Flow cytometry and cell sorting conducted in Examples are described below. Isolated AGM regions were dissociated by incubation with dispase (Boehringer) for 30 minutes at 37°C and cell dissociation buffer (Gibco-BRL) for 30 minutes at 37°C, followed by vigorous agitation to separate cells. Single cell suspensions of the AGM culture were prepared by incubating with cell dissociation buffer for 30 minutes at 37°C.

[0088] Cells were first incubated with 50 µl of mouse serum on ice for 30 minutes and biotinylated primary antibody was added at 10 µg/ml. After a 30 minute incubation on ice, a 20-fold volume of phosphate buffered saline at pH 7.4 (PBS) containing FCS was added and the cells were centrifuged. Cells were then incubated with allophycocyanin (APC)-conjugated streptavidin (Molecular probe, Eugene, OR) at 10 µg/ml for 30 minutes on ice with or without phycoerythrin (PE)-conjugated antibody. After washing with 5% FCS-PBS, cells were resuspended in 0.5 ml of PBS containing propidium iodide (PI) (Sigma, St. Louis, MO) and analyzed by FACS Calibur (Becton Dickinson). PI-positive dead cells were excluded. The monoclonal antibodies used for FACS were anti-CD45 (30F11.1), anti-Mac-1 (M1/70), anti-Gr-1 (RB6-8C5), anti-Thy-1.2 (30-H12), anti-B220 (RA3-6B2), anti-Ter-119 (TER-119), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD34 (RAM34), anti-CD31 (MEC13.3), anti-Fli1 (Avas12α1), and rat isotype control (R35-95), which were all purchased from Pharmingen. Anti-VECadherin antibody (VECD1) (Matsuyoshi, N. et al. (1997) Proc. Assoc. Am. Physicians 109, 362-371) was kindly provided by S. Nishikawa (Kyoto University).

[0089] For cell sorting, AGM regions from GFP positive embryos at 11.5 dpc were trypsinized as described above and cells (10<sup>7</sup>/ml) were incubated with biotinylated anti-PCLP1 antibody at 10 µg/ml in 5% FCS-PBS on ice for 30 minutes. After washing with 20-fold volumes of 5% FCS-PBS, cells were stained with PE-conjugated anti-CD45 antibody (10 µg/ml) and APC-conjugated streptavidin (10 µg/ml) on ice for 30 minutes and subjected to cell sorting using FACS Vantage. In a typical case of cell sorting in combination with anti-PCLP1 and anti-CD45 antibodies as described below, out of 1.1 x 10<sup>7</sup> cells obtained from 40 AGM regions, 8.5 x 10<sup>5</sup> of PCLP1<sup>+</sup>CD45<sup>+</sup> cells, 1.0 x 10<sup>6</sup> of PCLP1<sup>+</sup>CD45<sup>+</sup> cells, and 5.9 x 10<sup>4</sup> of PCLP1<sup>+</sup>CD45<sup>+</sup> cells were obtained by cell sorting.

#### [Example 1] Generation of hematopoietic cells in the AGM culture

[0090] The inventors' previous studies using an *in vitro* culture for AGM cells, they suggested that the endothelial-like cells in a AGM culture may contain hemangioblasts which give rise to hematopoietic progenitors *in vitro* (Mukouyama, Y. et al. (1998) Immunity 8, 105-114). Furthermore, timelap analysis of the cultured AGM cells under a phase contrast microscope showed that floating round cells with a hematopoietic appearance were spontaneously generated from adherent endothelial-like cells *in situ* (data not shown). To test the possibility that the adherent endothelial-like cells produced hematopoietic cells, the inventors examined the uptake of Dil-Ac-LDL, which is known to be incorporated only into endothelial cells and macrophages (Goldstein, J. L. et al. (1979) Proc. Natl. Acad. Sci. USA 76, 333-337; Voyta, J. C. et al. (1984) J. Cell Biol. 99, 2034-2040). As shown in Figure 1, the inventors first incubated AGM cells for 6 days to generate endothelial-like cells. AGM cells at day 6 were washed well with the culture medium to remove hematopoietic cells, and co-incubated with 10 µg/ml of 1,1'-dioctadecyl-3,3',3'-tetramethylindo-carbocyanine perchlorate (Biomedical Technologies, Inc., Stoughton, MA)-labeled acetylated low density lipoprotein (Dil-Ac-LDL) at

37°C for 6 hours. After washing twice with PBS, AGM cells were stained with anti-CD45 antibodies (Pharmingen, San Diego, CA) conjugated fluorescein isothiocyanate (FITC). Dil<sup>+</sup>CD45<sup>-</sup> cell population was sorted by FACS Vantage (Becton Dickinson, Bedford, MA) and inoculated to unlabeled AGM culture at day 6. Dil<sup>+</sup>CD45<sup>+</sup> hematopoietic cells appeared after 4 days of co-incubation (Figure 1). These Dil<sup>+</sup>CD45<sup>+</sup> hematopoietic cells were sorted and subjected to CFU-C assay.

[0091] CFU-C assay was conducted as follows: Cells (10<sup>4</sup>) were inoculated into 0.8% methylcellulose medium containing 20% fetal calf serum, IL-3 (100 ng/ml), IL-6 (kind gift from Ajinomoto, Kawasaki) (100 ng/ml), SCF (kind gift from Kirin Brewery, Takasaki, Japan) (100 ng/ml), and EPO (kind gift from Kirin Brewery) (2 U/ml) and cultured for 14 days as previously described (Mukouyama, Y. et al. (1998) *Immunity* 8, 105-114). These hematopoietic cells formed colonies in the CFU-C assay (data not shown), suggesting that some hematopoietic progenitor cells were derived from the Dil<sup>+</sup>CD45<sup>-</sup> endothelial-like cells in the AGM primary culture.

[Example 2] Preparation of monoclonal antibodies against surface antigens of endothelial-like cells derived from AGM culture

[0092] To define hemangioblasts more precisely, the inventors aimed to obtain a specific antibody directed against hemangioblasts. By repeating the passage of adherent cells of the AGM culture in the presence of OSM, the inventors were able to establish a novel OSM-dependent endothelial-like cell line, LO. LO cells exhibit characteristics very similar to those of endothelial-like cells in the AGM culture, such as endothelial-like morphology, incorporation of Dil-Ac-LDL, and production of hematopoietic cells. The inventors used the LO cells as immunogens to raise monoclonal antibodies against cell surface antigens on LO cells as follows.

[0093] Wistar rats (Nihon SLC) were immunized with 10<sup>7</sup> of LO cells in the presence of Freund's complete adjuvant (WAKO, Osaka, Japan) according to the standard immunization procedure (Hockfield, S. et al. (1993) "Selected Methods for Antibody and Nucleic Acid Probes", Volume 1 (New York: Cold Spring Harbor Laboratory Press)). Lymph nodes were dissociated and fused with mouse myeloma P3X cells using polyethylene glycol as previously described (Ogorochi, T. et al. (1992) *Blood* 79, 895-903) and hybridoma supernatants were screened for the production of anti-LO specific antibodies by FACS. 10B9 monoclonal antibody was chosen based on the specific staining of endothelial-like cells in the AGM culture. 10B9 antibody was produced in nude mice and purified by using E-Z-Sep (Pharmacia Biotech, Uppsala, Sweden). The isotype of the 10B9 antibody was determined by using the rat IgG isotyping kit (Serotec, Oxford, UK). Biotinylated 10B9 antibody was prepared by using Enzotin (Enzo Diagnostics, Syosset, NY) according to the manufacturer's instruction.

[0094] Flow cytometry revealed that the antibody designated 10B9 (rat IgG1) exhibited very clear staining of LO cells (Figure 2A) but not of NIH3T3 cells (data not shown). This antibody also stained endothelial-like cells in the AGM culture as described below (see Figure 5).

[Example 3] Molecular cloning of mouse PCLP1 molecule as a possible hemangioblast antigen

[0095] Next, using a standard expression cloning strategy with COS7 cells and 10B9 monoclonal antibody, the inventors isolated a cDNA clone encoding the 10B9 antigen.

[0096] Expression cloning of a cDNA encoding the 10B9 antigen was carried out by using COS7 cells as previously described (Harada, N. et al. (1990) *Proc. Natl. Acad. Sci. USA* 87, 857-861) except that magnetic beads conjugated with anti-rat IgG antibody (Dynabeads M-450) (Dyna, Oslo, Norway) were employed instead of plate panning. Briefly, COS7 cells were fused with spheroplasts of the cDNA plasmid library of LO cells (Tanaka, M. et al. (1999) *Blood* 93, 804-815) and stained with 10B9 antibody followed by Dynabead selection. Plasmid DNA mixture was harvested from the beads, amplified in *E. coli* and re-transfected into COS7 cells. This procedure was repeated 4 to 5 times until a single band of cDNA insert was recovered. As a result, the inventors isolated a cDNA clone of 1.9 kilobases encoding the 10B9 antigen.

[0097] DNA sequencing revealed that the C-terminal amino acid sequence was highly homologous to those of human and rabbit podocalyxin-like protein 1 (PCLP1) (Kershaw, D. B. et al. (1997) *J. Biol. Chem.* 272, 15708-15714; Kershaw, D. B. et al. (1995) *J. Biol. Chem.* 270, 29439-29446), suggesting that it was a mouse counterpart of PCLP1 (Figure 3). The avian PCLP1 homolog, thrombomucin, also shares the conserved regions (McNagny, K. M. et al. (1997) *J. Cell Biol.* 138, 1395-1407) (Figure 3). To obtain the full length mouse PCLP1 cDNA, the inventors isolated 5' cDNA fragments of mouse PCLP1 through screening of the original cDNA library and rapid amplification of the cDNA ends (RACE) method. 5'-RACE was performed using the 5'-RACE kit (GIBCO-BRL). The DNA sequences of the cDNAs were determined by using a Dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA) and an automated DNA sequencer (Applied Biosystems, Foster City, CA). The cDNA nucleotide sequence of mouse PCLP1 and the amino acid sequence of protein encoded by the cDNA are set forth in SEQ ID NO: 1 and 2, respectively.

[0098] COS7 cells were transfected with the reconstructed full length mouse PCLP1 cDNA in the pME18S expression

vector and were stained with 10B9 antibody. The COS7 cells transfected with PCLP1 cDNA exhibited specific staining with 10B9 antibody (Figure 2B), confirming that the 10B9 antibody recognizes mouse PCLP1. PCLP1 is an extensively glycosylated protein with a single transmembrane region. As previously reported (Kershaw, D. B. et al. (1997) J. Biol. Chem. 272, 15708-15714; Kershaw, D. B. et al. (1995) J. Biol. Chem. 270, 29439-29446), the amino acid sequence of the N-terminal region of PCLP1 is poorly conserved among species (Figure 3). Interestingly, a recent report suggested that both PCLP1 and CD34 are ligands for L-selectin in the high endothelial venule and that PCLP1 and CD34 share common amino acid sequences in their cytoplasmic tails (Sasseti, C. et al. (1998) J. Exp. Med. 187, 1965-1975). These homologous amino acid residues are also found in mouse PCLP1 at positions 440 to 451, 464 to 473 and 500 to 503 (Figure 3).

**[0099]** For Northern blotting, poly(A)<sup>+</sup> RNA samples were electrophoretically separated in 1.0% agarose gel and transferred onto a nylon membrane (Boehringer Mannheim, Mannheim, Germany). The RNA was then hybridized with digoxigenin (DIG) -labeled single strand DNA probe for the PCLP1 cDNA (2.1 kb) as described previously (Tanaka, M. et al. (1999) Blood 93, 804-815)

**[0100]** PCLP1 was originally identified as a major component of podocytes in the rabbit kidney and demonstrated to be expressed in some endothelial cells (Kershaw, D. B. et al. (1995) J. Biol. Chem. 270, 29439-29446). Consistent with previous reports (Kershaw, D. B. et al. (1997) J. Biol. Chem. 272, 15708-15714; Kershaw, D. B. et al. (1995) J. Biol. Chem. 270, 29439-29446), the inventors detected PCLP1 mRNA in kidney, heart, lung, brain, and muscle, but not in spleen, thymus, small intestine, or liver of adult mice (Fig. 4). The same size of mRNA was also detected in LO cells (Figure 4). The avian counterpart of PCLP-1, thrombomucin, was reported to be expressed in thrombocytes and multipotent hematopoietic progenitors (McNagney, K. M. et al. (1997) J. Cell Biol. 138, 1395-1407). Likewise, expression of PCLP1 was found in some bone marrow cells (data not shown) and hematopoietic cells in the AGM region (see Figure 6, 8A) as described below.

#### [Example 4] Expression of PCLP1 on the endothelial-like cells in the AGM culture

**[0101]** The inventors examined the expression of PCLP1 on the endothelial-like cells in the AGM culture by immunostaining with 10B9 anti-PCLP1 antibody. Cultured AGM-derived cells in plastic plates were fixed with 1% paraformaldehyde (PFA)-PBS at room temperature for 15 minutes and incubated with anti-PCLP1 10B9 antibody at 10 µg/ml at 4°C over night. After incubation with peroxidase-conjugated anti-rat IgG (Amersham), signals were visualized by 3,3'-diaminobenzidine (DAB) as previously described (Hara, T. et al. (1998) Dev. Biol. 201, 144-153).

**[0102]** As the inventors expected, PCLP1 was detectable on endothelial-like cells (Figure 5A to C), but not on fibroblastic cells (data not shown) in the AGM culture. The endothelial-like cells, defined by their polygonal cell morphology and incorporation of Dil-Ac-LDL, were further fractionated by fluorescent activated cell sorting (FACS) using anti-PCLP1 and anti-CD45 antibodies (Figure 6). Except for the erythroid lineage, CD45 is known to be a pan specific marker for hematopoietic cells including LTR-HSCs (Morrison, S. J. et al. (1995) Annu. Rev. Cell. Dev. Biol. 11, 35-71). It is noteworthy that hematopoietic cells (CD45<sup>+</sup>) in the AGM culture also express a high level of PCLP1 (Figure 6) as is the case for the AGM region (Figure 8A).

**[0103]** To examine whether the PCLP1<sup>+</sup>CD45<sup>-</sup> non-hematopoietic fraction contains hemangioblasts, PCLP1<sup>+</sup>CD45<sup>-</sup> cells (2 x 10<sup>5</sup>) were isolated (the R2 gate shown in Figure 6) from the day 6 AGM culture of transgenic mice expressing green fluorescent protein (GFP) and were inoculated into the day 6 AGM culture of nontransgenic mice. After 4 days of incubation, GFP<sup>+</sup>CD45<sup>+</sup> hematopoietic cells appeared in both floating and adherent fractions (Figure 7), indicating that hematopoietic cells are generated from the PCLP1<sup>+</sup>CD45<sup>-</sup> endothelial-like cells in the AGM primary culture. The adherent GFP<sup>+</sup>CD45<sup>+</sup> cells may represent the hematopoietic cells present underneath the stromal cell layer.

#### [Example 5] Expression and localization of PCLP1 in the AGM region of a mouse embryo

**[0104]** The inventors next examined the presence of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells in the intact AGM region of mouse embryos at 11.5 dpc. Based on FACS staining, there were more PCLP1<sup>+</sup>CD45<sup>-</sup> cells (32%) than PCLP1<sup>+</sup>CD45<sup>+</sup> cells (1.5%) in the AGM region (Figure 8A). The sorted PCLP1<sup>+</sup>CD45<sup>-</sup> cells were adherent cells with the capacity to incorporate Dil-Ac-LDL (data not shown), indicating that these cells are endothelial-like cells. It was recently reported that hematogenic angioblasts in the yolk sac and the P-Sp region express Flk1, VECadherin, and CD34 (Nishikawa, S. I. et al. (1998) Immunity 8, 761-769). Thus, the inventors examined whether these molecules are expressed in the PCLP1<sup>+</sup>CD45<sup>-</sup> fraction. Interestingly, a majority of nonhematopoietic CD34<sup>+</sup> cells, CD31<sup>+</sup> cells, and Flk1<sup>+</sup> cells also expressed PCLP1 (Figure 8B, 9C), whereas 79% of VECadherin<sup>+</sup>CD45<sup>-</sup> cells were found in the PCLP1<sup>+</sup> fraction (Figure 9D). Consistent with the overlapping expression patterns of PCLP1 and CD34, expression of these two proteins was localized in the endothelium of the dorsal aorta (Figure 10E, 10H) and in the genital ridge region (Figure 10F, 10I) of the mouse embryo at 11.5 dpc.

**[0105]** *In situ* hybridization analysis of the paraffin sections of a mouse embryo was conducted as follows. For prep-

aration of paraffin sections, the caudal half of mouse embryos at 11.5 dpc was fixed in 4% PFA-PBS for 10 hours. Paraffin sections (6 µm thick) were prepared as previously described (Hara, T. et al. (1998) Dev. Biol. 201, 144-153) and placed on poly-L-lysine-coated slide glasses. After hydration of paraffin sections, the sections were stained with anti-PCLP1 or anti-CD34 antibody at 10 µg/ml at 4°C over night and visualized as described above. Samples were counterstained with methylgreen.

**[0106]** *In situ* hybridization of the paraffin sections was carried out as previously described (Imakawa, K. et al. (1995) Endocrine 3, 511-517). DIG-labeled antisense and sense RNA probes were prepared by using the 5'-part of the PCLP1 cDNA fragment (nucleotide 126 to 354).

**[0107]** *In situ* hybridization analysis of the PCLP1 mRNA in the paraffin sections of a mouse embryo also revealed a similar expression pattern in the dorsal aorta (Figure 11N) and the genital ridge (Figure 11O). Since endothelial-like cells in the day 6 AGM culture do not express VECadherin and CD34 (data not shown), the inventors employed PCLP1 as a marker for the separation of hemangioblasts in the AGM region in the following Examples.

#### [Example 6] Endothelial differentiation of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells from the AGM region

**[0108]** The PCLP1<sup>+</sup>CD45<sup>-</sup> cell fraction was separated by cell sorting from the AGM region of mouse embryos at 11.5 dpc (the R1 gate in Figure 12). The sorted cells were reanalyzed, but CD45<sup>+</sup> cells were undetectable (Figure 12). Even 3 hours after plating of the sorted PCLP1<sup>+</sup>CD45<sup>-</sup> cells, no hematopoietic-like cells could be detected by microscopic observation and the cells were capable of incorporating Dil-Ac-LDL (data not shown). When these cells were cultured in the presence of OSM for 6 days, endothelial-like cells increased by 10-folds during incubation (Figure 13), incorporated Dil-Ac-LDL and expressed Flk1 (Figure 14 top and bottom), whereas no cells grew in the absence of OSM. Since only 12% of the sorted PCLP1<sup>+</sup>CD45<sup>-</sup> cells were Flk1<sup>+</sup> cells at the time of separation (Figure 9C), Flk1<sup>+</sup> cells may be selectively expanded or Flk1 expression may be induced during cultivation. The PCLP1<sup>+</sup>CD45<sup>-</sup> cells grown in the presence of OSM were partially positive for CD31, negative for CD34, and very weakly positive for VECadherin (Figure 15).

**[0109]** To test the possibility that the PCLP1<sup>+</sup>CD45<sup>-</sup> cells differentiate to endothelial cells, the inventors employed the OP9 co-culture system that has been used to induce endothelial differentiation *in vitro* (Hamaguchi, I. et al. (1999) Blood 93, 1549-1556; Hirashima, M. et al. (1999) Blood 93, 1253-1263).

**[0110]** Mouse calvaria-derived OP9 cells (kindly provided by S. Nishikawa, Kyoto University) were passaged as previously described (Kodama, H. et al. (1994) Exp. Hematol. 22, 979-984). Sorted PCLP1<sup>+</sup>CD45<sup>-</sup> cells from the AGM region were inoculated on subconfluent OP9 cells in a AGM culture medium containing various cytokines and cultured for 10 days. For the generation of hematopoietic cells, 5 x 10<sup>4</sup> cells were co-cultured with OP9 in the presence of SCF (100 ng/ml), bFGF (1 ng/ml), LIF (10 ng/ml), OSM (10 ng/ml), IL-3 (10 ng/ml), and EPO (2 U/ml). For endothelial cell differentiation, 10<sup>4</sup> cells were co-cultured in the presence of OSM (10 ng/ml), bFGF (1 ng/ml), and VEGF (PeproTech, London, UK) (10 ng/ml).

**[0111]** For matrigel assays, cells (2 x 10<sup>5</sup>) were resuspended in Dulbecco's modified Eagle's medium containing 1% fetal calf serum and VEGF (10 ng/ml) and overlaid on a Biocoat matrigel basement membrane (Becton Dickinson) in a 6-well plate. After 12 hours in culture, network formation was microscopically observed.

**[0112]** The PCLP1<sup>+</sup>CD45<sup>-</sup> cells from the AGM region were co-cultured with OP9 stromal cells for 10 days in the presence of OSM, VEGF, and bFGF as described above. The resultant cells expressed higher levels of CD34 and VECadherin (Figure 15) than those in the initial cell population (Figure 8B, 9D) or those cultured with OSM alone. Co-culture of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells with OP9 also resulted in an increased expression of CD31 and a decreased expression of PCLP1 (Figure 15). Moreover, the OP9 co-cultured cells formed a vascular network on a matrigel plate (Figure 16), while the cells grown without OP9 failed to form a network (data not shown). These results indicate that PCLP1<sup>+</sup>CD45<sup>-</sup> cells in the AGM region are able to differentiate to endothelial cells in the presence of OP9, OSM, VEGF, and bFGF. Therefore, PCLP1<sup>+</sup>CD45<sup>-</sup> cells are likely to be the endothelial precursor cells, i.e. angioblasts. Growth of the angioblasts in the AGM region appears to be OSM-dependent and their differentiation requires additional factors including VEGF, bFGF, and unknown factors produced from OP9 cells.

**[0113]** On the other hand, co-culture of PCLP1<sup>+</sup>CD45<sup>-</sup> cells with OP9 cells in the presence of hematopoietic growth factors containing SCF, interleukin (IL)-3, and erythropoietin (EPO) resulted in the development of hematopoietic cells. The hematopoietic cells included Mac-1/Gr-1 positive myeloid cells, B220/Thy-1-positive lymphoid cells, and Ter119-positive erythroid cells (Figure 17), suggesting that multiple lineages of hematopoietic cells were generated from the PCLP1<sup>+</sup>CD45<sup>-</sup> cells *in vitro*. Generation of these hematopoietic cells was also OSM-dependent. Taken together with the data from the Dil-Ac-LDL labeling experiment (Figure 1), it can be concluded that PCLP1<sup>+</sup>CD45<sup>-</sup> cells in the AGM region contain hemangioblasts and angioblasts.

#### [Example 7] Generation of LTR-HSCs from the PCLP1<sup>+</sup>CD45<sup>-</sup> cells in the AGM region

**[0114]** A major goal of this Example was to know whether hemangioblasts in the AGM region could give rise to

LTR-HSCs *in vivo*. LTR-HSCs were detected among the hematopoietic progenitors expanded in the AGM culture by the standard repopulation assay using irradiated adult mice. However, it was revealed that they are more efficiently engrafted when injected into livers of busulfan-treated neonatal mice (data not shown). This is reasonable as LTR-HSCs generated in the AGM region seed the fetal liver *in vivo* before homing into the bone marrow. The inventors thus considered the possibility that if LTR-HSCs were generated from the hemangioblasts present in the AGM region, they would engraft the neonatal liver more efficiently than in irradiated adult mice, and verified that. According to a recently established procedure (Yoder, M. C. et al. (1996) Biol. Blood Marrow Transplant. 2, 59-67), the inventors injected the PCLP1<sup>+</sup>CD45<sup>-</sup> cells from the AGM regions of GFP transgenic mouse embryos at 11.5 dpc into the busulfan-treated nontransgenic neonatal mice.

[0115] Transplantation of cells into busulfan-treated neonatal mice was performed as previously described (Yoder, M. C. et al. (1997) Immunity 7, 335-344) with a slight modification. Briefly, busulfan (Sigma) was intraperitoneally injected into pregnant C57BL/6 mice at 12.5 µg/g on pregnant day 17 and 18. Within 24 to 48 hours after birth, cells derived from GFP mice in 25 µl of PBS were injected into the liver of neonatal mice. Peripheral blood of recipient mice was taken at 2 or 6 months after transplantation and analyzed for GFP chimerism.

[0116] As summarized in Table 1, donor-derived GFP positive hematopoietic cells were detected in the peripheral blood of 7 out of 9 mice at 2 to 6 months after the injection of the GFP<sup>+</sup>PCLP1<sup>+</sup>CD45<sup>-</sup> cells. To repopulate the donor-derived blood cells, an injection of  $1.7 \times 10^5$  cells or more was required, indicating that a small fraction of the PCLP1<sup>+</sup>CD45<sup>-</sup> cell population are capable of generating LTR-HSCs. In contrast, no donor-derived hematopoietic cells were found by injecting the same number of PCLP1<sup>+</sup>CD45<sup>-</sup> cells from the AGM region (the R2 gate in Figure 12) (Table 1). Moreover, injection of the PCLP1<sup>+</sup>CD45<sup>+</sup> cells ( $0.9$  to  $2.1 \times 10^4$ ) did not contribute to GFP chimerism, which effectively excluded the possibility that a small number of contaminating CD45<sup>+</sup> cells in the PCLP1<sup>+</sup>CD45<sup>-</sup> fraction repopulated in the recipient mice. The chimerism was maintained up to 6 months in both myeloid and lymphoid compartments of the peripheral blood of a mouse (10B3 mouse) injected with  $1.7 \times 10^5$  of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells (Figure 18). In the 10B3 mouse, all lineages of donor-derived GFP positive hematopoietic cells were repopulated in the spleen (Figure 19) and bone marrow (Figure 20). CD4/CD8-double positive and mature single positive T cells derived from the donor were also detected in the recipient thymus (Figure 21). These results indicate that hemangioblasts with potential to generate LTR-HSCs are present in the PCLP1<sup>+</sup>CD45<sup>-</sup> cell population of the AGM region. Although a similar number of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells derived from the day 6 AGM primary culture showed a decreased repopulation potential (Table 1), they were capable of generating hematopoietic cells *in vitro* (Figure 7). Hence, the *in vitro* culture of the AGM cells negatively affected the repopulation potential of the PCLP1<sup>+</sup>CD45<sup>-</sup> cells.

Table 1

Hematopoietic cell generation from PCLP1 <sup>+</sup> CD45 <sup>-</sup> cells in engrafted mice				
Exp.	Cell	Fraction	Cell number/mouse	Engrafted/Total (% Chimerism)
1	AGM	PCLP1 <sup>+</sup> CD45 <sup>-</sup>	$1.7 \times 10^5$	1/1* (53%)
		PCLP1 <sup>+</sup> CD45 <sup>+</sup>	$9.0 \times 10^3$	0/1*
2	AGM	PCLP1 <sup>+</sup> CD45 <sup>-</sup>	$2.8 \times 10^5$	2/2 (21%, 1.2%)
		PCLP1 <sup>+</sup> CD45 <sup>+</sup>	$2.8 \times 10^5$	0/3
3	AGM	PCLP1 <sup>+</sup> CD45 <sup>-</sup>	$3.4 \times 10^5$	1/1 (29%)
		PCLP1 <sup>+</sup> CD45 <sup>+</sup>	$3.4 \times 10^5$	0/1
4	AGM	PCLP1 <sup>+</sup> CD45 <sup>-</sup>	$2.2 \times 10^5$	3/5 (4.4%, 2.6%, 1.5%)
		PCLP1 <sup>+</sup> CD45 <sup>+</sup>	$2.1 \times 10^4$	0/4
5	AGM culture	PCLP1 <sup>+</sup> CD45 <sup>-</sup>	$3.0 \times 10^5$	2/5 (0.35%, 0.23%)
		PCLP1 <sup>+</sup> CD45 <sup>+</sup>	$2.9 \times 10^4$	0/2
6	AGM culture	PCLP1 <sup>+</sup> CD45 <sup>-</sup>	$3.0 \times 10^5$	2/5 (0.14%, 0.14%)
		PCLP1 <sup>+</sup> CD45 <sup>+</sup>	$3.0 \times 10^5$	0/2

[Each cell fraction was sorted from AGM region or AGM culture of GFP mice and injected into busulfan-treated neonatal mice. Peripheral blood was taken at 2 months (6 months for those marked "\*\*") after injection and subjected to FACS analysis. Relative frequency of GFP<sup>+</sup> cells in engrafted mice was calculated and expressed as "% chimerism".]

Industrial Applicability

**[0117]** The present invention provides a method for preparing a cell fraction containing hemangioblasts capable of generating both endothelial cells and hematopoietic cells, and a marker molecule "PCLP1" for hemangioblasts utilized in the preparation. A cell fraction according to the present invention is capable of not only differentiating into endothelial-like cells and hematopoietic cells, but also of expressing a long-term hematopoietic function *in vivo*. The method of this invention enables the screening and separation of hemangioblasts in various tissues and cells. A cell fraction of this invention is not only useful, for example, in screening factors and drugs that regulate the proliferation and differentiation of hematopoietic stem cells, but could also be used for isolating novel cell markers for hemangioblasts and hematopoietic stem cells, or for screening antibodies used in cell sorting.



## SEQUENCE LISTING

5 <110> Center for Advanced Science and Technology  
Incubation, Ltd.

<120> Method for preparing cell fraction containing hemangioblasts

10 <130> SEN-113PCT

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       Trp Ile Val Pro Leu Asp Asn Leu Thr Lys Asp Asp Leu Asp Glu Glu  
    485                                490                                495

30     gaa gac aca cac ctc tgatctgtct gctggccacc ccaactgcac tgcagagctc 1719  
       Glu Asp Thr His Leu  
       500

35     cagaccaccc aaagtgccat ttggacaggg aggggggaaat cttcccacct gaagacagaa 1779  
       agactgggga gggagaatgg actccgaagg ttgtctccct cctcacaatc cctacagggc 1839  
       cttaattttc cttttcaagt tcaacaaagt cacattctgt ctagtccct catgtaaaat 1899

40     aacctacttg tgctgagcc cagagcagga aggcaggagg aagatggaga acgcatgcg 1959  
       agtctctgga gctcctccag gaacggcacc ctctcatctc ataactgagg ccttaagagg 2019  
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45     ggtgcttaat tgcattcaga cacactactg tgttcatctg tgccattcc cc 2131

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       <212> PRT  
       <213> Mus musculus

55     <400> 2  
       Met Pro Pro Thr Thr Ala Leu Ser Ala Leu Leu Leu Leu Leu Ser  
       1                                5                                10                                15

5

	260	265	270
5	Pro Ser Thr Pro Ser Pro Ile Trp Ala Phe Gly Asn Tyr Gln Leu Asn 275	280	285
10	Cys Glu Pro Pro Ile Arg Pro Asp Glu Glu Leu Leu Ile Leu Asn Leu 290	295	300
	Thr Arg Ala Ser Leu Cys Glu Arg Ser Pro Leu Asp Glu Lys Glu Lys 305	310	315 320
15	Leu Val Glu Leu Leu Cys His Ser Val Lys Ala Ser Phe Lys Pro Ala 325	330	335
20	Glu Asp Leu Cys Thr Leu His Val Ala Pro Ile Leu Asp Asn Gln Ala 340	345	350
	Val Ala Val Lys Arg Ile Ile Ile Glu Thr Lys Leu Ser Pro Lys Ala 355	360	365
25	Val Tyr Glu Leu Leu Lys Asp Arg Trp Asp Asp Leu Thr Glu Ala Gly 370	375	380
30	Val Ser Asp Met Lys Leu Gly Lys Glu Gly Pro Pro Glu Val Asn Glu 385	390	395 400
	Asp Arg Phe Ser Leu Pro Leu Ile Ile Thr Ile Val Cys Met Ala Ser 405	410	415
35	Phe Leu Leu Leu Val Ala Ala Leu Tyr Gly Cys Cys His Gln Arg Ile 420	425	430
40	Ser Gln Arg Lys Asp Gln Gln Arg Leu Thr Glu Glu Leu Gln Thr Val 435	440	445
	Glu Asn Gly Tyr His Asp Asn Pro Thr Leu Glu Val Met Glu Thr Pro 450	455	460
45	Ser Glu Met Gln Glu Lys Lys Val Val Asn Leu Asn Gly Glu Leu Gly 465	470	475 480
50	Asp Ser Trp Ile Val Pro Leu Asp Asn Leu Thr Lys Asp Asp Leu Asp 485	490	495
55	Glu Glu Glu Asp Thr His Leu 500		

## Claims

1. A method for preparing a cell fraction containing hemangioblasts, wherein said method comprises separating cells comprising a PCLP1-positive phenotype.
2. The method according to claim 1, which further comprises separating cells comprising a CD45-negative phenotype.
3. The method according to claim 1 or 2, wherein said cells are separated from cells derived from the aorta-gonad-mesonephros (AGM) region.
4. A PCLP1-positive cell fraction containing hemangioblasts that is prepared by a method according to any one of claim 1 through 3.
5. The cell fraction according to claim 4, which generates or contains long-term repopulating hematopoietic stem cells (LTR-HSCs).
6. A cell composition containing the cell fraction according to claim 4 and a culture medium.
7. A method for preparing a chimeric animal, wherein said method comprises transplanting the cell fraction according to claim 4.
8. A chimeric animal transplanted with the cell fraction according to claim 4.
9. The chimeric animal according to claim 8, wherein donor (transplanted cells)-derived blood cells can be reconstructed.
10. The chimeric animal according to claim 8 or 9, wherein said animal is mouse.
11. A DNA according to any one of the following (a) through (c), wherein the DNA encodes the mouse-derived PCLP1 protein:
  - (a) a DNA comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 1,
  - (b) a DNA encoding a protein comprising the amino acid sequence set forth in SEQ ID NO: 2, and
  - (c) a DNA encoding a protein comprising the amino acid sequence set forth in SEQ ID NO: 2 in which one or more amino acids are substituted, deleted, inserted, and/or added.
12. A protein encoded by the DNA according to claim 11.
13. A vector into which the DNA according to claim 11 has been inserted.
14. A host cell carrying the vector according to claim 13.
15. A method for preparing the protein according to claim 12, wherein said method comprises the steps of culturing the host cell according to claim 14 and collecting expressed proteins from said host cell or culture supernatant thereof.
16. An antibody against PCLP1 protein, wherein the antibody is used for detecting or separating a cell fraction containing hemangioblasts.
17. The antibody according to claim 16 that binds to a protein as defined in claim 12.
18. A separation reagent for a cell fraction containing hemangioblasts, wherein said reagent comprises the antibody according to claim 16 or 17.
19. An antibody that specifically binds to mouse-derived PCLP1 protein.
20. An antibody that binds to a peptide comprising the amino acid residues at positions 1 to 405 in the amino acid sequence set forth in SEQ ID NO: 2.



21. A peptide containing a partial sequence comprising at least 7 or more consecutive amino acid residues at positions 1 to 405 in the amino acid sequence set forth in SEQ ID NO: 2.

22. A polynucleotide comprising at least 15 nucleotides, wherein the polynucleotide is complementary to DNA comprising the nucleotide sequence set forth in SEQ ID NO: 1 or to the complementary strand thereof, and is used in the amplification and detection of the expression of the DNA according to claim 11, or in the expression control of the DNA.

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Figure 1

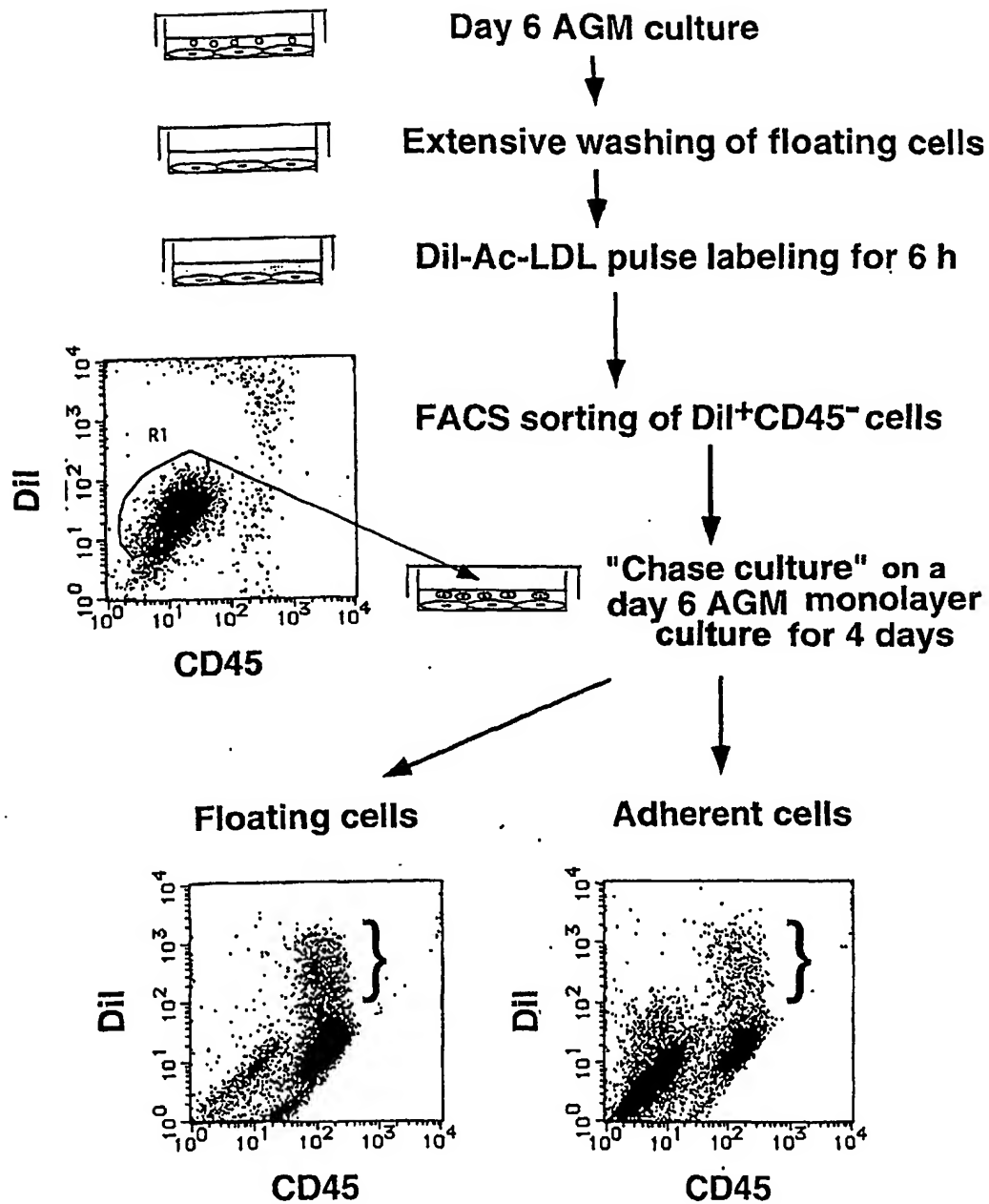


Figure 2

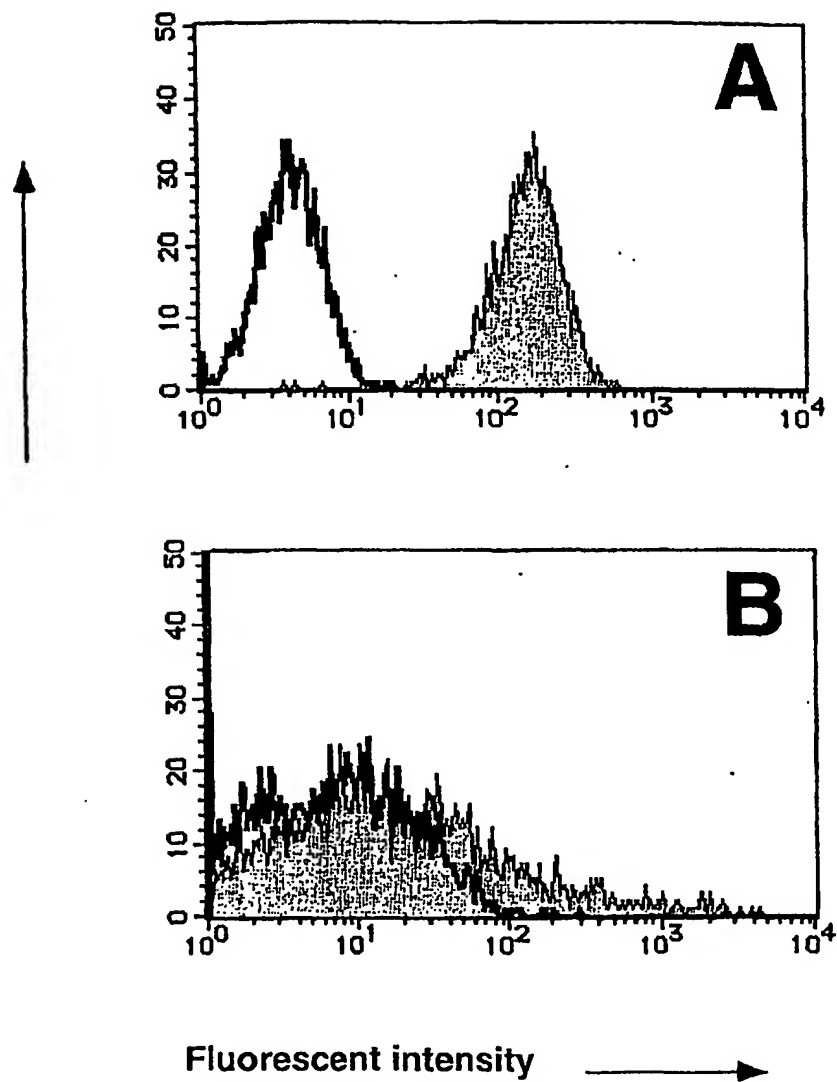


Figure 3

mouse PCLP1	1: MPPTTALSALLLLSPASHSH-NGNETSTSAIKSST-VQSHQSATTSTEVTTGHPVAST	58
human PCLP1	1: MRCALALBAL-LLLSTPP-LL-PSSPSPSPSP-SPSQ-NA-TQTTD-SSNKTAPTPAS	53
rabbit PCLP1	1: MRSALALALALLLLSPPSLSQEKSPQPGTPMATSTSTRPAPASAPAPKSSVAASVPAE	60
Thrombomucin	1: MRAPLLPLPLPLPGVSSGNDKTHSTTVSPETTKQITITVTTSSQVQGSISASKPSS	60
mouse PCLP1	59: LAST-QPSNTP-FT-TSTQSPFMPSTPNP-TSNQSGGNLTSVSEVDKTKTSSPSSTA	114
human PCLP1	54: SV-T-IMATDTAQSTVPTSKANEILASV--KATTL--GVSSDSP-GTTTLAQQVS-GP-	104
rabbit PCLP1	61: QNTT-PMTTKAP-ATQSPSASPGSSVENSAP-AQGSTTTQQSLSVTTKAEKADAGGVPTA	117
Thrombomucin	61: TAPTAVMSFTKAQEAATSSKQHDSSSTSIIPPSTSIPTSIITTSPOGKTPSTPALHTPTD	120
mouse PCLP1	115: FTSSSGQTASSGGK-SG-DSFTT-APTT-TL-G-----L--INVSSQ---PTDLNNTSKL	159
human PCLP1	105: VNT-TVARGGGSGN-P-TTTIESPK-STK-SAD--TTTVA-TSTAT-AK-PNNTSSQ---	151
rabbit PCLP1	118: HVTGSARPVTSGSQVAAQDPAASKAPSNHSITTKP---LA-TEATSQ--APRQTTDVGTP	171
Thrombomucin	121: QNTKTTGRQDDTSHVSVASTSASQVSSSASAAVPTTSAVTSSATQQKVSPDTSSEILL	180
mouse PCLP1	160: LST--PTDNTSPQQPVDSSPSTASHP--VG-QHTPAAV-PSSSGSTPSTDNSTLTWKP	213
human PCLP1	152: N-GAE-DTN-SG-GKSSHS-VTDLTSTK-AEHLTP-HP-TSPLSPRQ-P--T-L--T	197
rabbit PCLP1	172: GPTA-PPVTNSTSPDLLGHATPKPSEGP-QLSFPTAAGSLGPVTGSGTGSLSTPQGKP	229
Thrombomucin	181: KPSASPNTQVTSPTSPTPKGFLSTVTTSPHIDNGSTALNQLKSTVSSSEVPVSSFLDKD	240
mouse PCLP1	214: TTHKPLGTSEATQ-----PL--TSQTP-GITTLVVS-TLQQSM-ASTVGTT-TEEFT-H-	260
human PCLP1	198: HPVATPTSSGHDHLMKISSSSSTVAIPGYTFTSP--G-MTTLPSSVISQRTQQTSSQMP	254
rabbit PCLP1	230: ATLTPVASSAETQGM-PSMPSPSPAS-SSSPFPSSPSPSPALQPSGSPAAGTEDTTGRC	287
Thrombomucin	241: HSVSSSTSATNQL-SLSSHRPTSPVPKFECSPTHSGSVPTSSKTSLSPPSSSTKNATV	299
mouse PCLP1	261: LISNGTPVAP-P--GPSTPSPIWAFGNVQLNCEP-PIRPDBELL-ILN--LTR--ASLC-	310
human PCLP1	255: ASSTAPSSQETVQPTSPATALRPTLPETMSSSPTAASTTHRYPTPSPTVAHESNNAKC	314
rabbit PCLP1	288: PTSSTELASTALHGPSTLSPTSFAVRDQRVSCGP-PERPTEQLL-ILN--LTR--SSPCI	341
Thrombomucin	300: TTMTTAKAAYTSQGDGSVTHKSGVTAQSPTSAPLPTPTLKDHMKSKSPDQTHSNVSPPN	359
mouse PCLP1	311: ---ER-S-----PLD--EK---E-KLVELLCHSVKASFKAEDLCTLHVAPI	347
human PCLP1	315: EDLETQTQSEKQLVLNLTGNTLCAG--GASDEKLISLICRAVKATFNPAQDKCGIRLASV	372
rabbit PCLP1	342: HVPQRQSQGE-GET-BI---SMHSTDLS-PEDKLVTLICRAAKPTFNPAQDQCHVLLAPM	395
Thrombomucin	360: EVICEDQIGEVRIPLNLKEEKTCDWKKASNEAFFEVFCSGRRHAFNSTRDCTVKLAS-	418
mouse PCLP1	348: LDNQAVAPKRIIETKLSPKAYKELKDRWDDLTEAGVSDMKLGKEGPPPEVNEORFBLPL	407
human PCLP1	373: PGSQTVVYKEITHTKLPAKDYKELKDKWDELKEAGVSDMKLGDQGPPEABDRFBLPL	432
rabbit PCLP1	396: LGSHAVVYKEITIKTNLLPTAVFELKDRWDDLREBGVSDMKLGDQGPPEBEDRFBLPL	455
Thrombomucin	419: SNHRRWAV-HVIVHRVLDPAAVFEELEKERNLEKLGTINVTYLNQEMEEBIKQSSSTPL	477
mouse PCLP1	408: ITIVCMASFLLVLAALYGCCHQRLSQRKQQRLTEELQTVENGYHDNPTLEVMTGSEM	467
human PCLP1	433: ITIVCMASFLLVLAALYGCCHQRLSQRKQQRLTEELQTVENGYHDNPTLEVMTGSEM	492
rabbit PCLP1	456: ITIVCMASFLLVLAALYGCCHQRLSHRKQQRLTEELQTVENGYHDNPTLEVMTGSEM	515
Thrombomucin	478: ITIVFLAGSLLLAALYGCCHQRFSSKKSQRLTEELQTVENGYHDNPTLEVMTGSEM	537
mouse PCLP1	468: QEKVVNLNGELGDSWIVPLDNLTKDPLDEEDTHL	503
human PCLP1	493: QEKVVNLNGELGDSWIVPLDNLTKDPLDEEDTHL	528
rabbit PCLP1	516: QEKVVNLNGELGDSWIVPLDNLTKDPLDEEDTHL	551
Thrombomucin	538: QEKK-VNLNGELGDSWIVPLDTIMKEDL-DEEDTHL	571

Figure 4

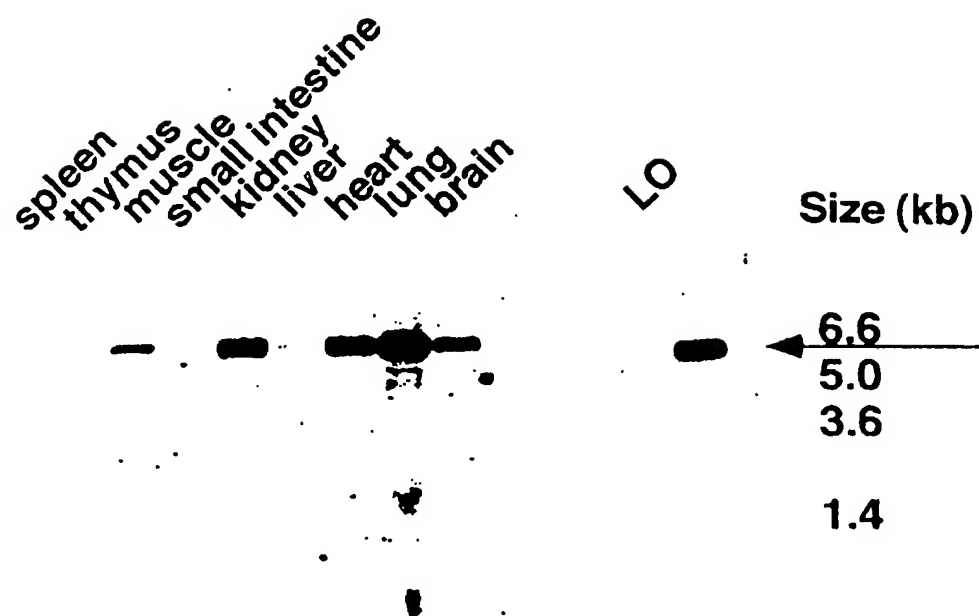


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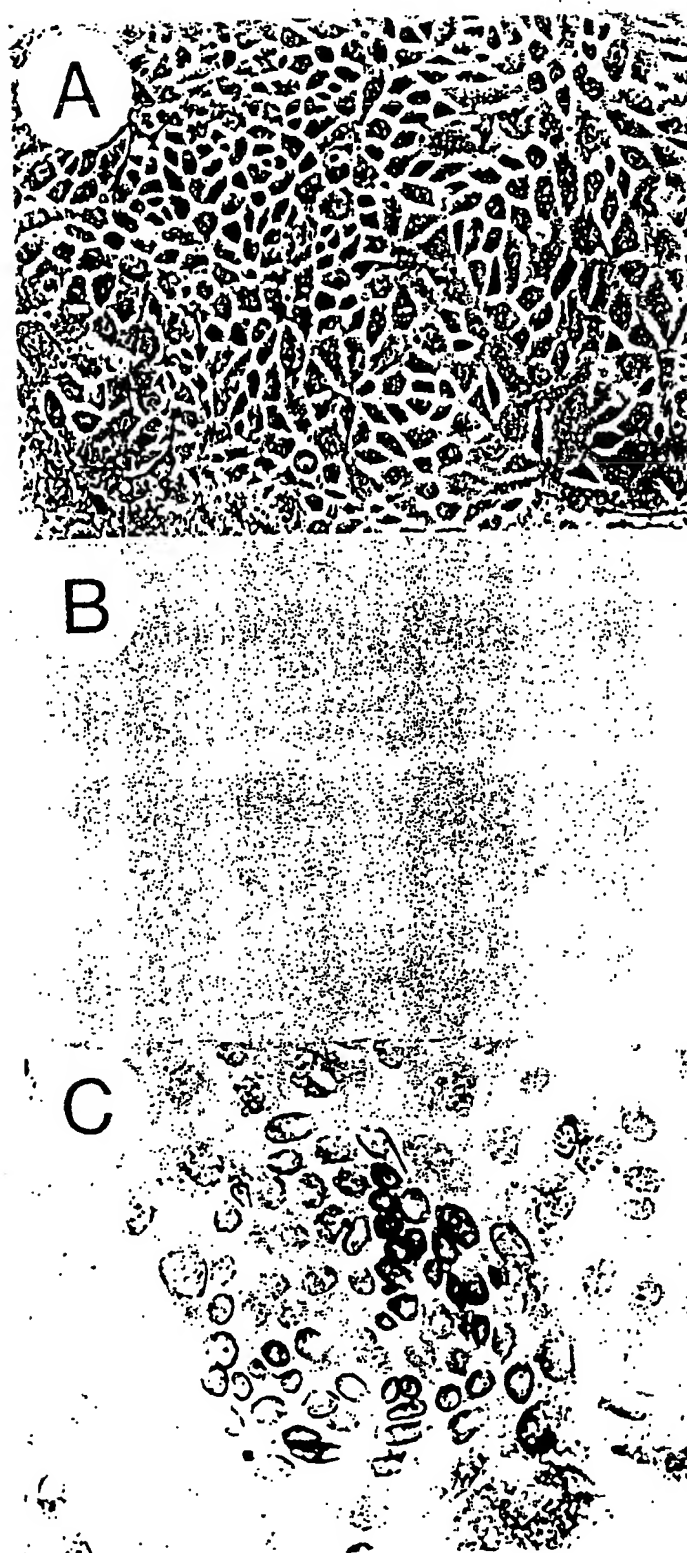


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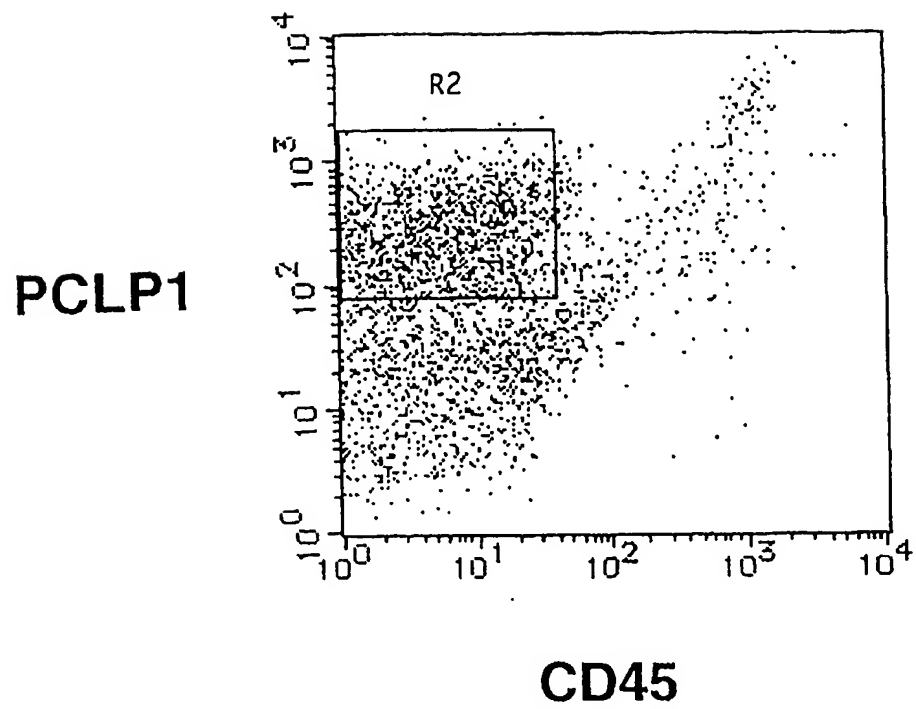




Figure 7

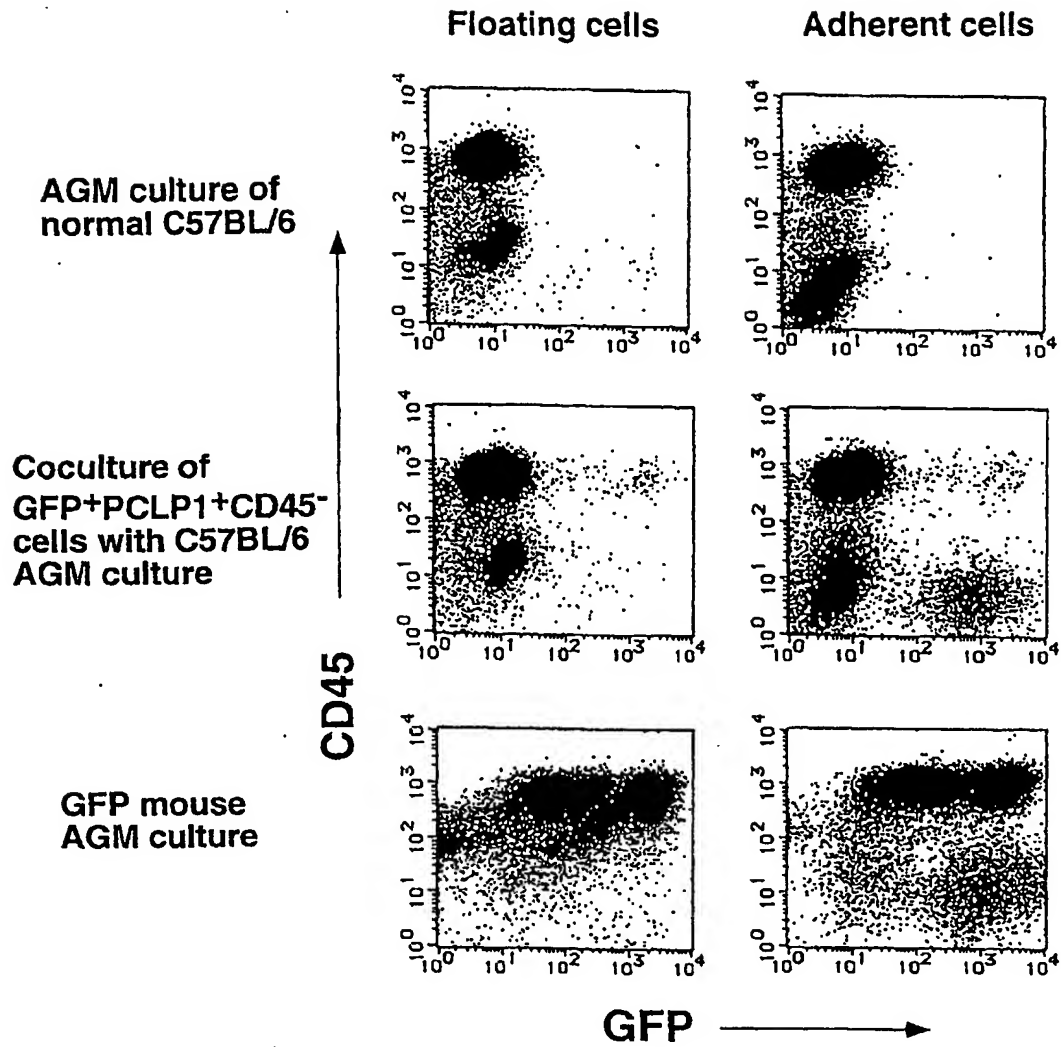


Figure 8

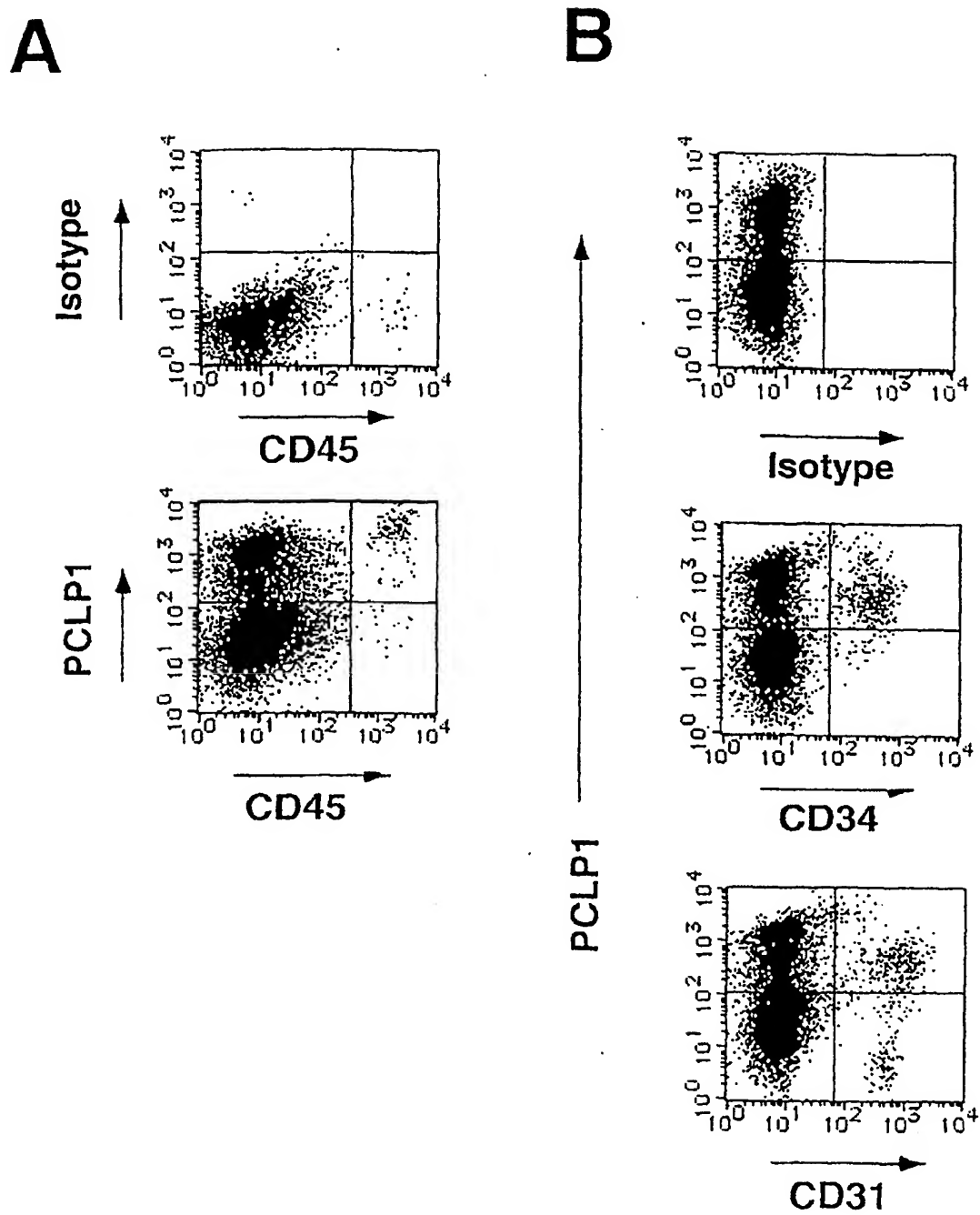
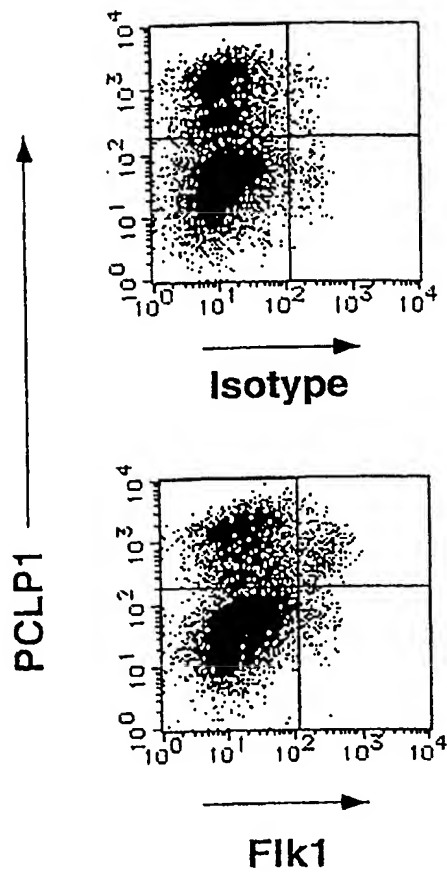


Figure 9

**C**



**D**

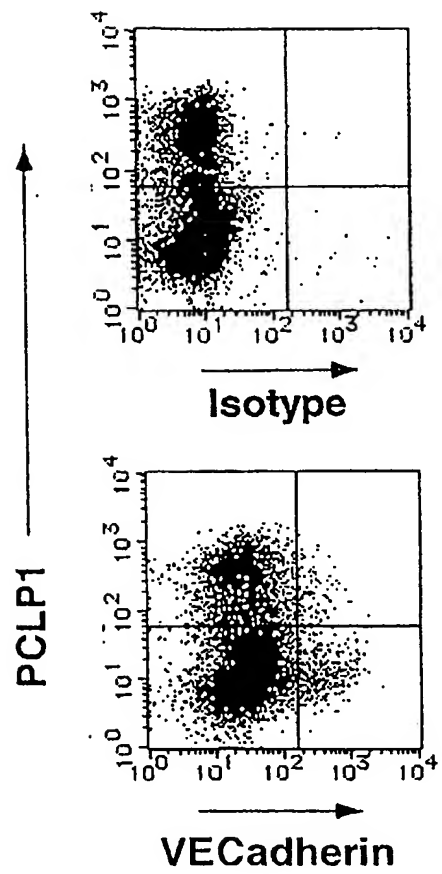


Figure 10

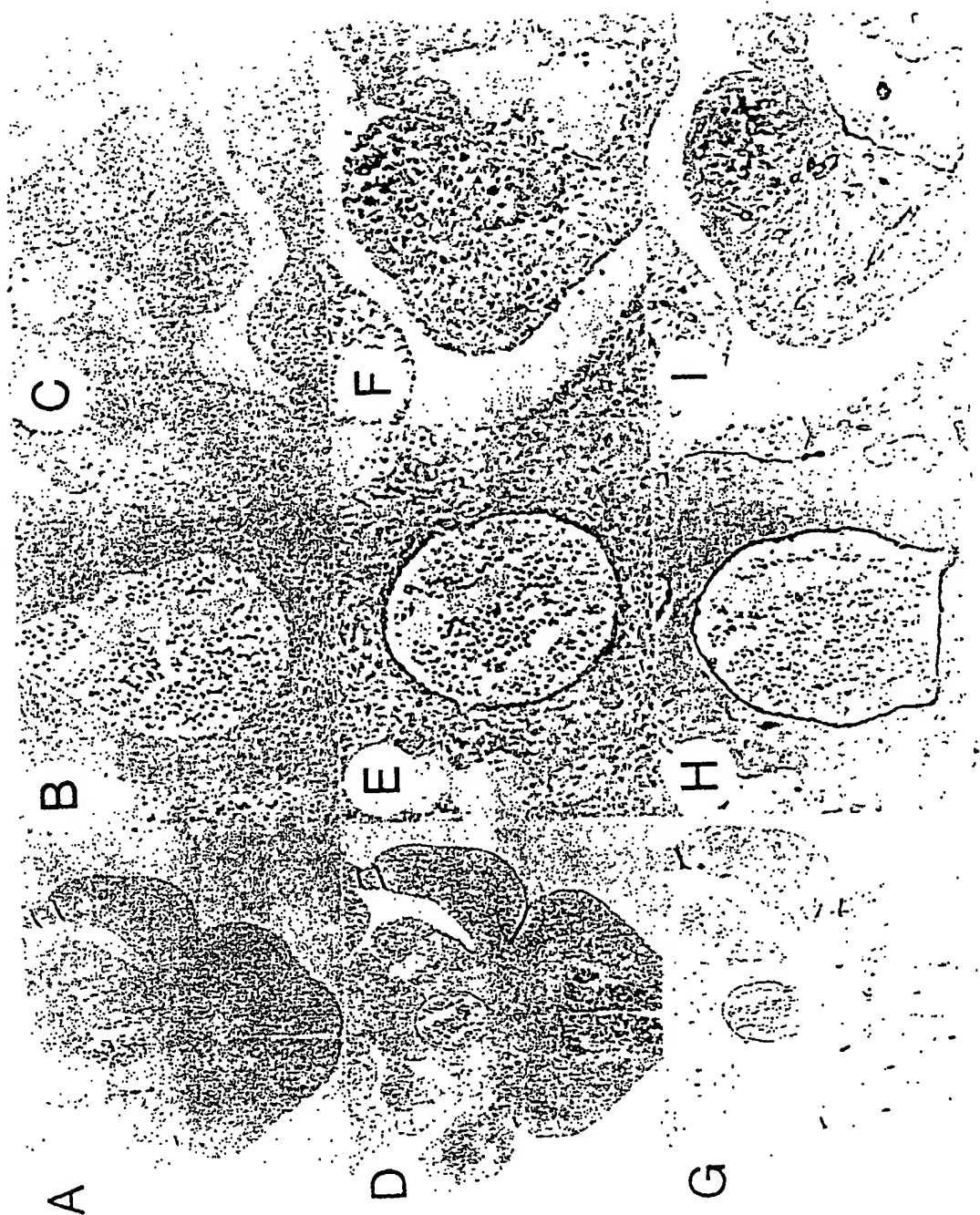


Figure 11

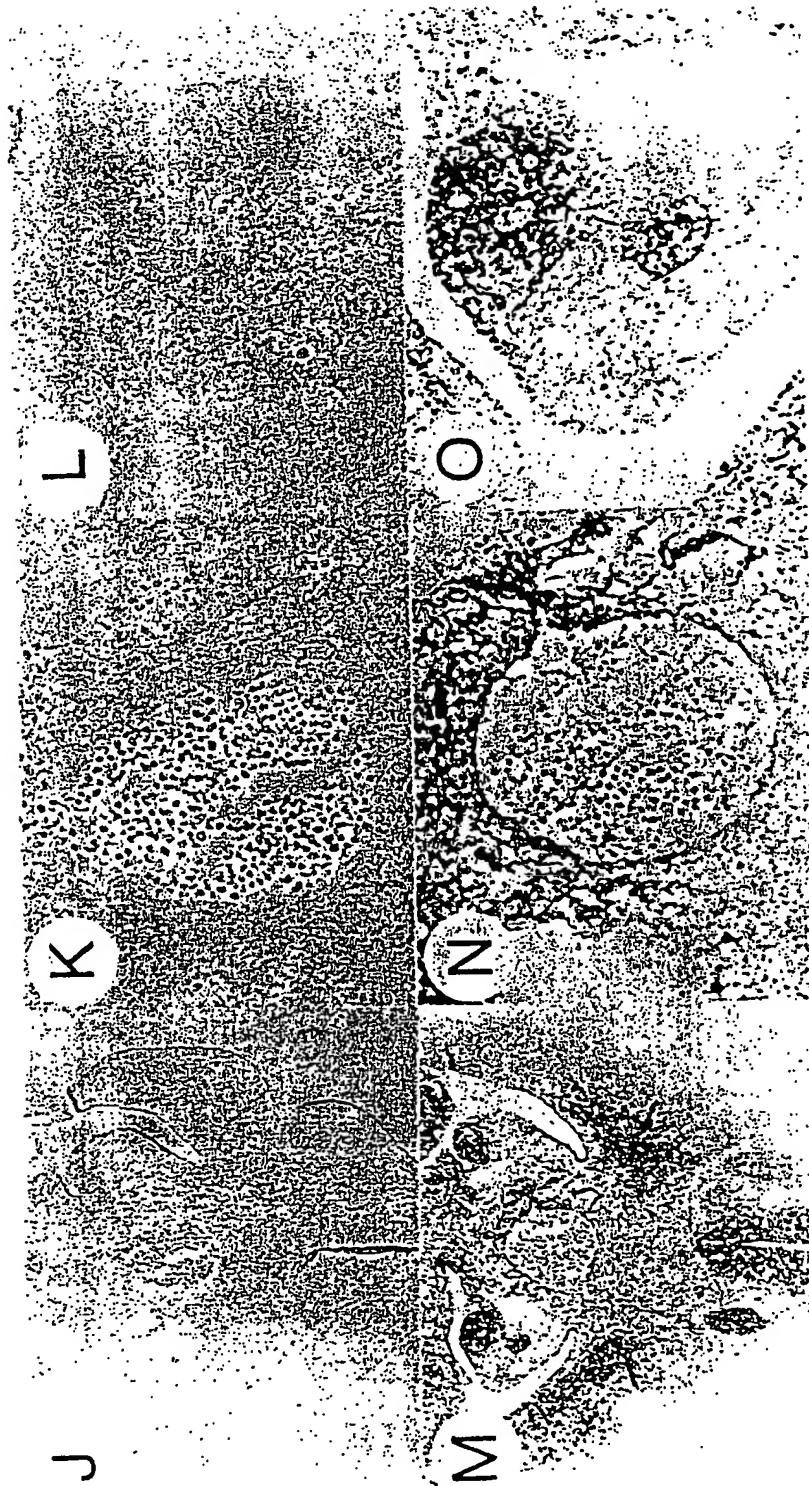


Figure 12

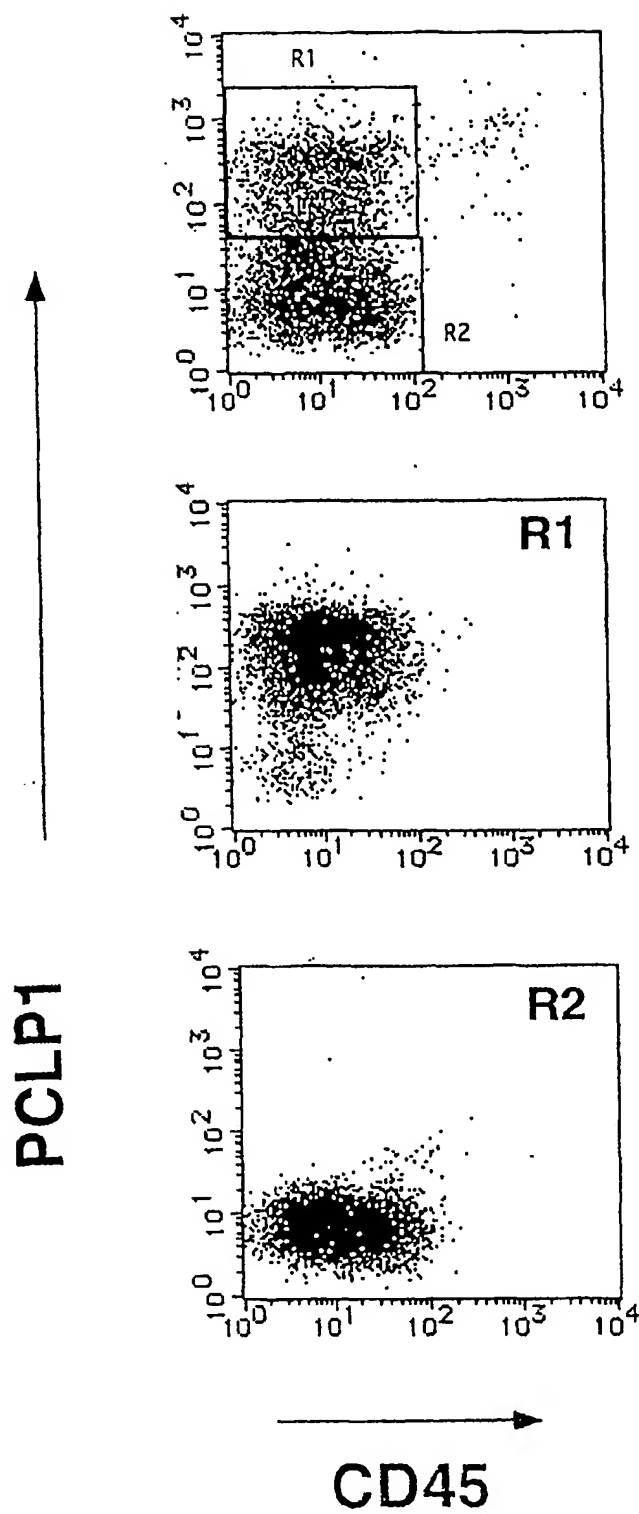


Figure 13

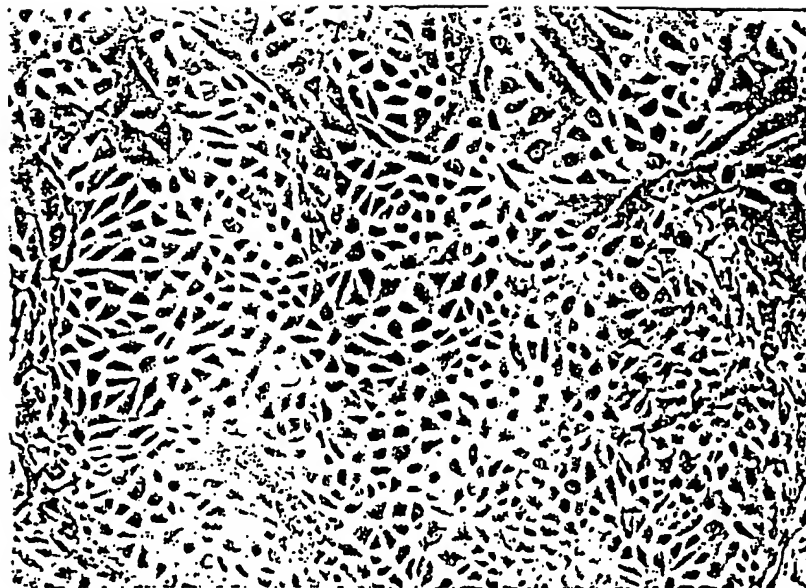




Figure 14

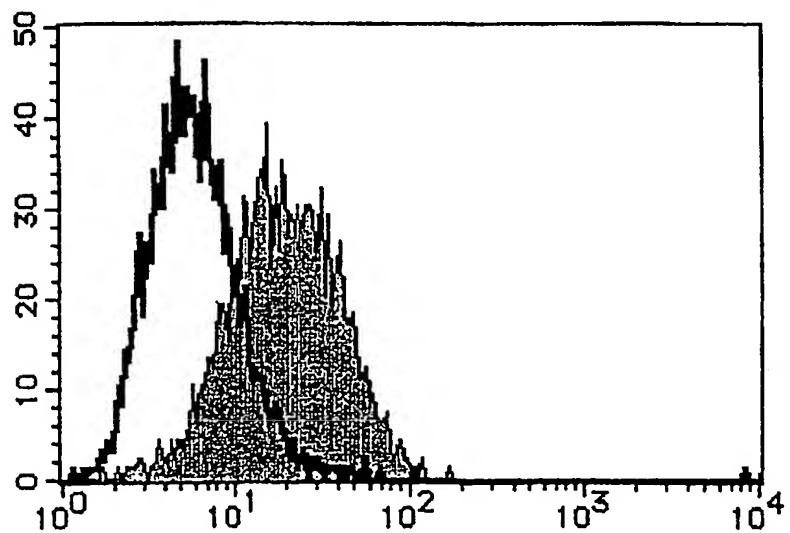
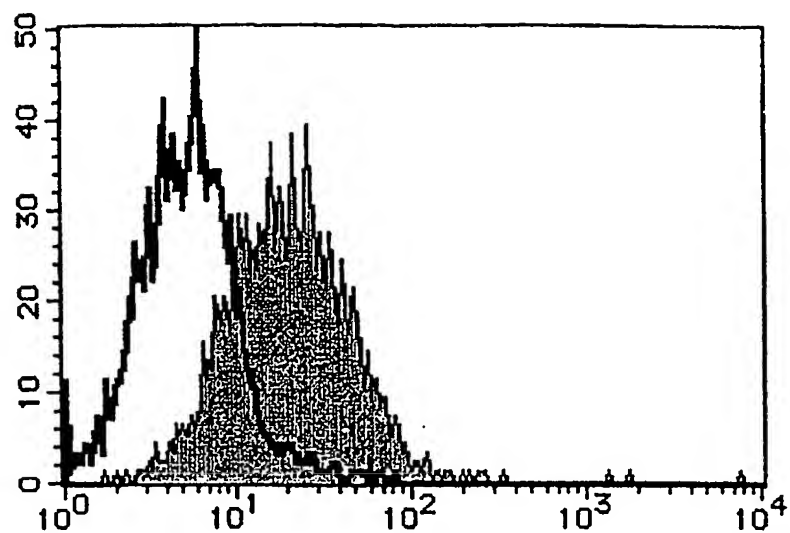


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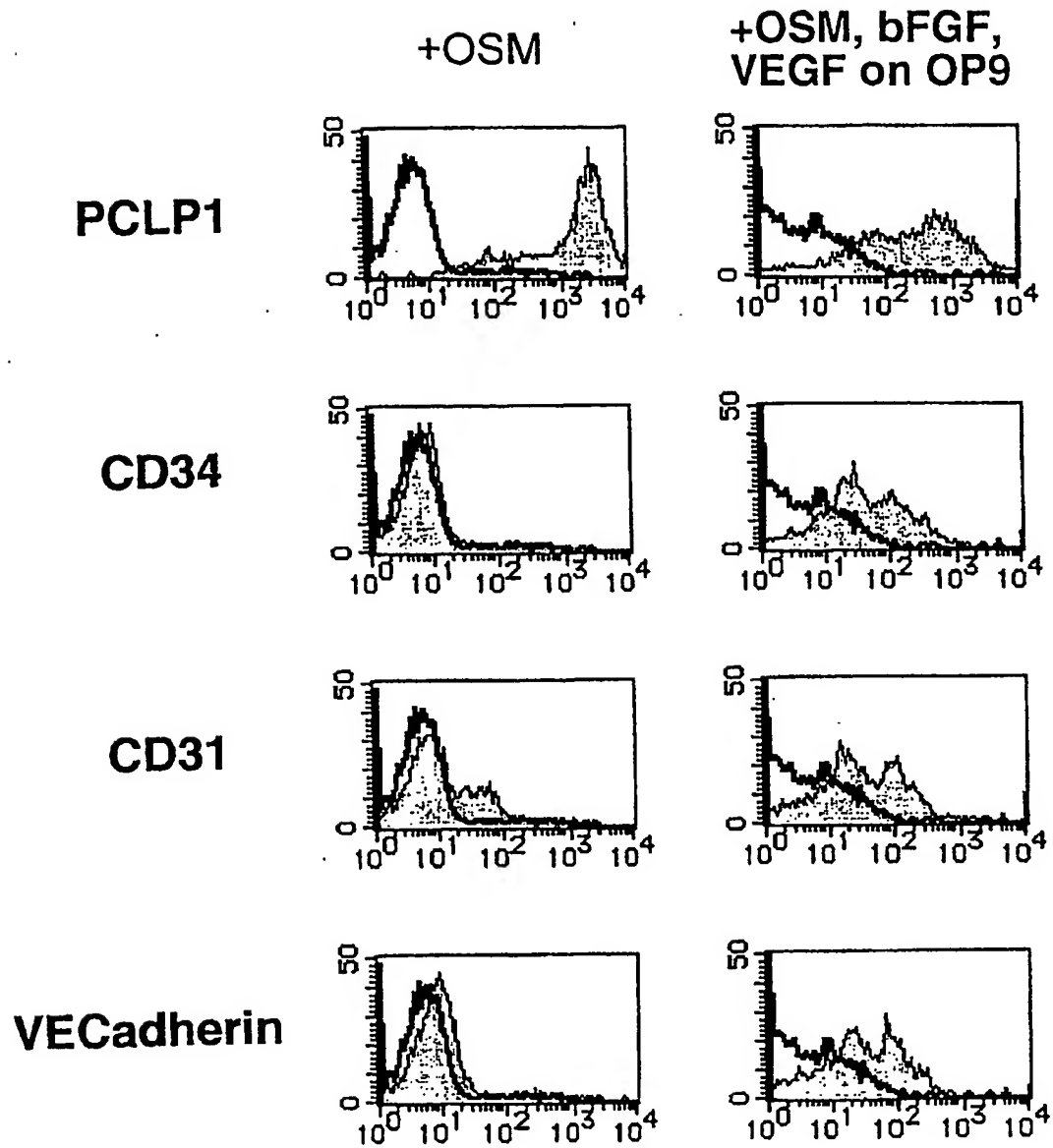


Figure 16



Figure 17

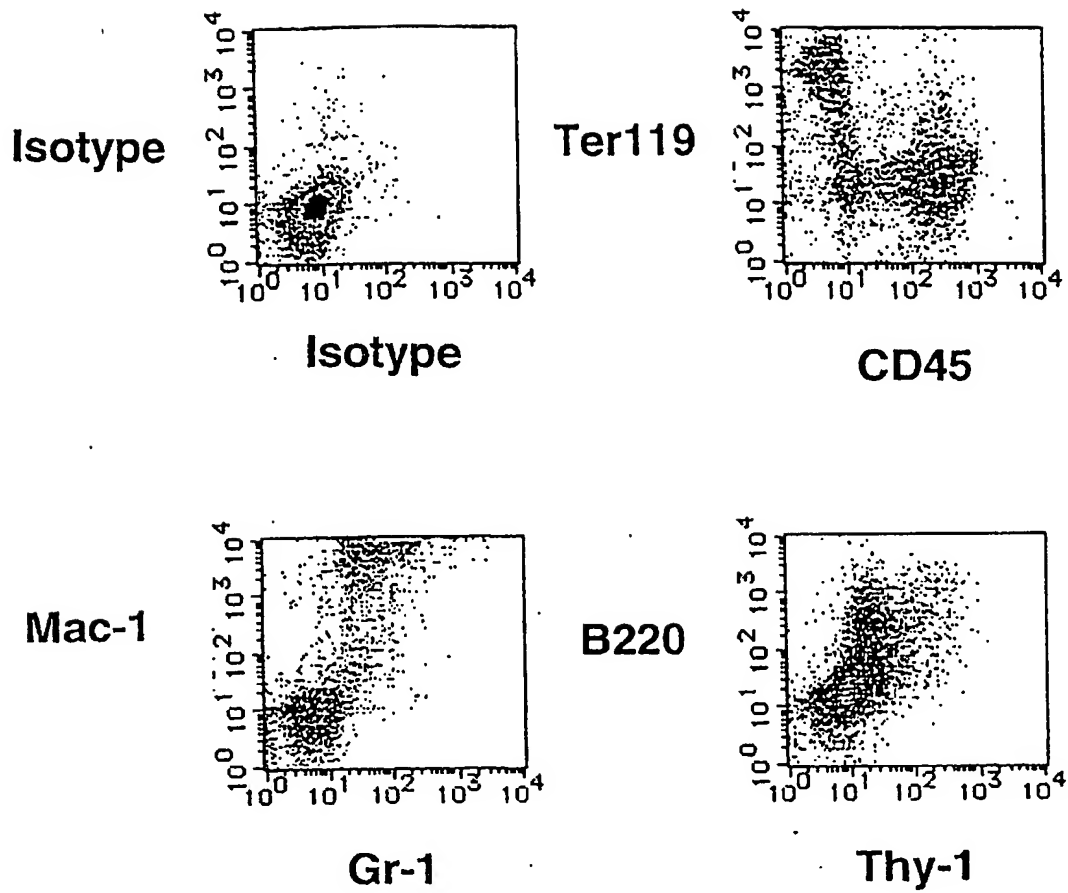


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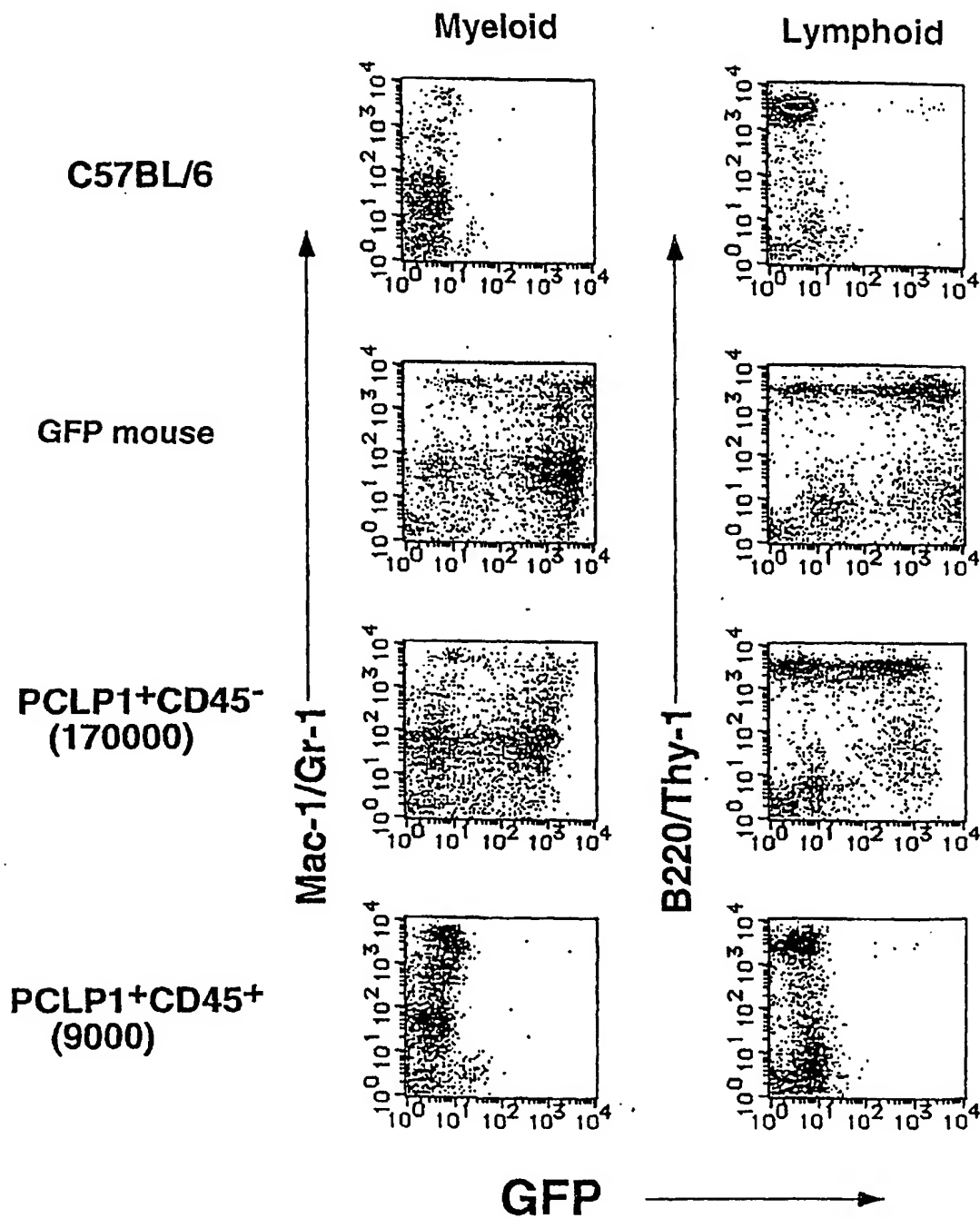


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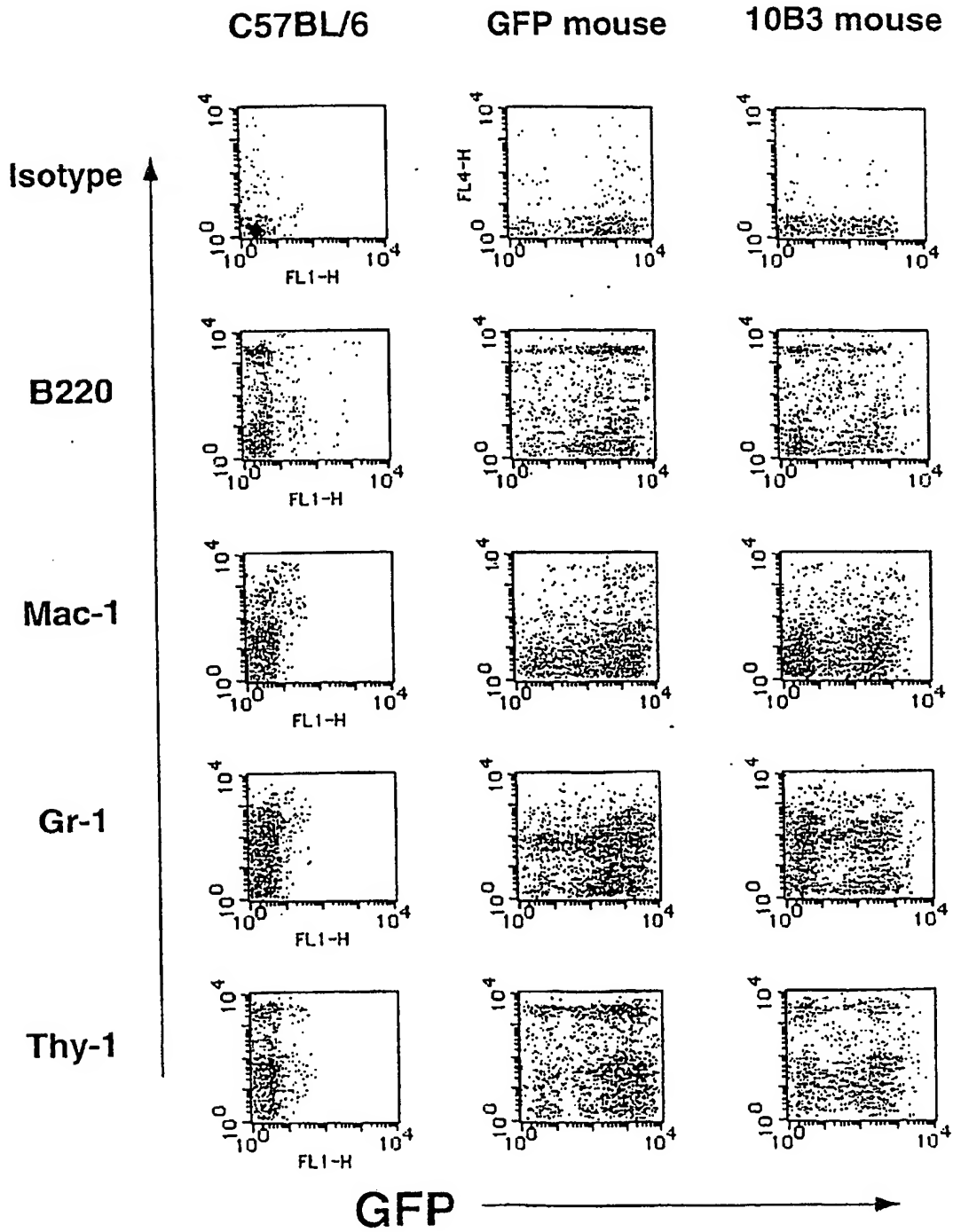


Figure 20

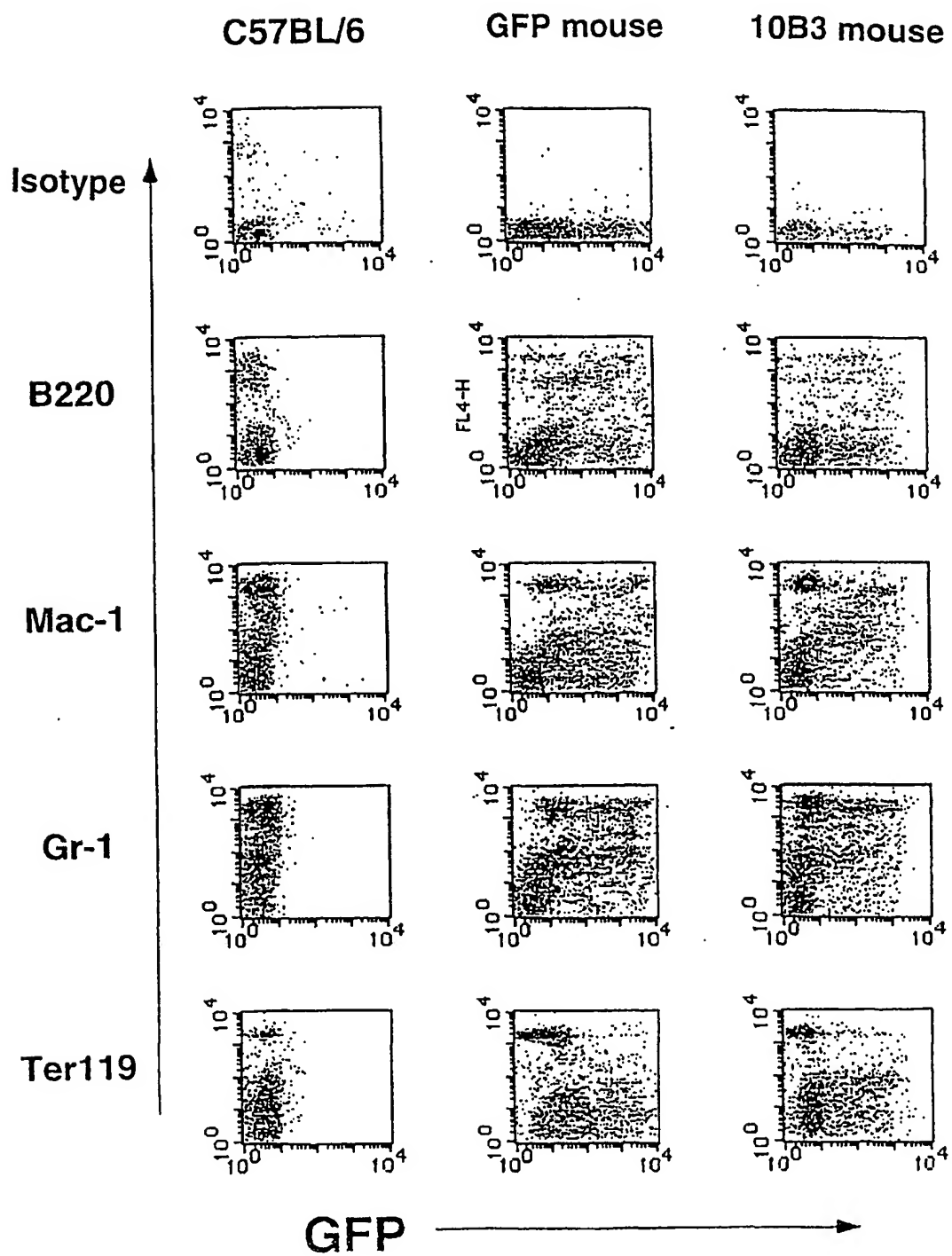
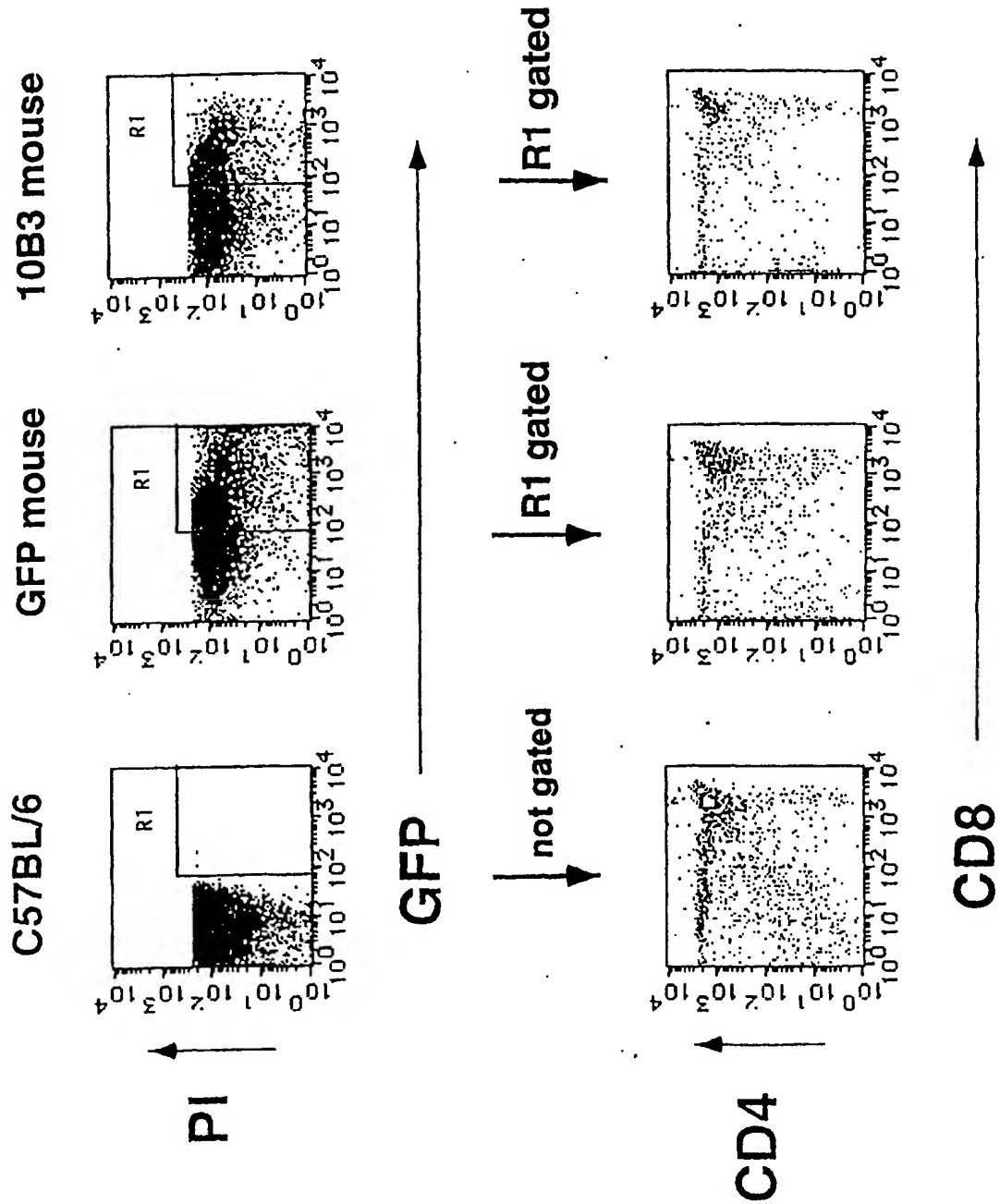


Figure 21





## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/07817

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int.Cl. <sup>7</sup> C12N15/12, C12N5/00, C07K14/47, C07K16/18, C12P21/08, C12Q1/68, A01K67/027		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) Int.Cl. <sup>7</sup> C12N15/12, C12N5/00, C07K14/47, C07K16/18, C12P21/08, C12Q1/68, A01K67/027		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) MEDLINE (STN) Genbank/EMBL/DDBJ/GeneSeq		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	David B. Kershaw et al., "Molecular Cloning, Expression, and Characterization of Podocalyxin-like Protein 1 from Rabbit as a Transmembrane Protein of Glomerular Podocytes and Vascular Endothelium" J. Biol. Chem., Vol. 270, No. 49, pp. 29439-29446 (1995)	11-15, 19-22
X	David B. Kershaw et al., "Molecular Cloning and Characterization of Human Podocalyxin-like Protein" J. Biol. Chem., Vol. 272, No. 25, pp. 15708-15714 (1997) Hara T. et al.,	11-15, 19-22
P, X	"Identification of podocalyxin-like protein 1 as a novel cell surface marker for hemangioblasts in the murine aorta-gonad-mesonephros region" Immunity, Vol. 11, pp. 567-578 (1999)	1-22
A	Nishikawa S. et al., "Progressive lineage analysis by cell sorting and culture identifies FLK1+ VE-cadherin+ cells at a diverging point of endothelial and hemopoietic	1-22
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
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Date of the actual completion of the international search 30 January, 2001 (30.01.01)		Date of mailing of the international search report 06 February, 2001 (06.02.01)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/07817

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	lineages" Development, Vol.125, pp.1747-1757 (1998)	

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